The role of Bone Morphogenetic Protein (BMP) signaling in human granulosa cells during the periovulatory interval

by

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Abstract

Ovulation is a complex and finely-tuned process whereby a series of closely-regulated events happen, including cumulus-oocyte complex (COC) expansion, resumption of oocyte meiosis and follicle rupture. These events must occur before luteinization; otherwise the oocytes are trapped within follicles and destined to degenerate. Appropriate timing requires specific changes in granulosa cell functions that are governed by gonadotropins and regulated by intrafollicular molecular regulators.

In animal models, bone morphogenetic proteins (BMPs) are essential for successful ovulation by inhibiting early luteinization. However, the detailed functions and underlying mechanisms of BMPs on the ovulation/luteinization process have not been fully established. In the present research, immortalized (SVOG) and primary human granulosa cells were used to investigate the effects of recombinant human BMPs on steroid hormones and COC expansion-related protein production. Receptor-mediated signaling was investigated using activin receptor-like kinase (ALK) inhibitors and small interfering RNAs targeting ALKs and SMADs.

In human granulosa cells, BMP4 and BMP7 suppress progesterone production and steroidogenic acute regulatory protein (StAR) expression while increasing estradiol production and aromatase expression. This fact suggests that BMPs could inhibit premature luteinization by decreasing the progesterone/estradiol ratio. BMPs (BMP4, BMP6, BMP7 and BMP15) up-regulate hyaluronan synthases 2 (HAS2) expressions and hyaluronan production as well as the expression of the hyaluronan binding protein versican. BMP4 also induced A Disintegrin and Metalloproteinase with TromboSPondin Repeats-1 (ADAMTS-1) protein levels and activity of versican proteolytic cleavage. It suggests that BMPs could promote COC mass formation while simultaneously loosing the matrix by increasing versican cleavage. In addition, the findings that

the effects of BMPs on different gene expressions are differentially mediated by ALK inhibitors suggest that human granulosa cells have the capacity to signal via multiple BMP type I receptors in response to these factors, but that the receptor(s) are differentially coupled to specific target genes or cellular responses. Moreover, BMPs regulate different biological processes by activating different SMAD signaling: down-regulate progesterone production through canonical SMAD1/5/8 signaling, while up-regulate hyaluronan production through noncanonical SMAD2/3 signaling. These findings deepen knowledge of the physiological roles of BMPs during periovulatory interval and may lead to applying to *in vitro* fertilization (IVF) protocol improvement.

Preface

A version of chapter 3 has been accepted by *Endocrinology*. **Han Zhang**, Christian Klausen, Hua Zhu, Hsun-Ming Chang, Peter C.K. Leung. BMP4 and BMP7 suppress StAR and progesterone production via ALK3 and SMAD1/5/8-SMAD4 in human granulosa cells.

A version of chapter 4 has been prepared and expected to be submitted. **Han Zhang**, Tian Shen, Christian Klausen, Hua Zhu, Peter C.K. Leung. Differential activation of noncanonical SMAD2/ SMAD3 signaling by bone morphogenetic proteins causes disproportionate induction of hyaluronan production in human granulosa cells.

My supervisor Dr. Peter C.K. Leung, my committee member Dr. Christian Klausen and I designed the experiments in chapter 3, 4, and 5. I conducted all the experimental procedures in chapter 3, 4 and 5. Dr. Shen Tian and Dr. Hua Zhu assisted me in primary granulosa cells culture. Dr. Hsun-Ming Chang designed some primers and validated the antibodies.

All my studies in this thesis were approval by the University of British Columbia Research Ethics Board. Certificate Number: H90-70337

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3β-HSD	3β-Hydroxysteroid dehydrogenase/Δ5-Δ4 isomerases
17β-HSD	17β-Hydroxysteroid dehydrogenase
ACVR	Activin receptor
ADAMTS	A distingrin and metalloproteinase with thrombospondin motifs
ALK	Activin receptor-like kinase
AMH	Anti-müllerian hormone
AMHR	AMH receptor
ANOVA	Analysis of variance
AV	Atrioventricular
BMP	Bone morphogenetic protein
BMPR-2	BMP type II receptor
Bp	Base pair
Ca^{2+}	Calcium
cAMP	Cyclic adenosine monophosphate
cDNA	Complementary deoxyribonucleic acid homolog
C/EBP	CCAAT/enhancer binding protein
cGMP	Cyclic guanosine monophosphate
CL	Corpus luteum
Co-SMAD	Common SMAD
COC	Cumulus-oocyte-complex
COH	Controlled ovarian hyperstimulation

COX2	Cyclooxygenase-2
CRE	cAMP-response elements
CREB	CRE-binding protein
dNTP	Deoxynucleoside triphosphate
DMEM	Dulbecco's Modified Eagle's Medium
DNA	Deoxynucleic acid
DNase	Dexyribonuclease
EC	Endocardial cushion
ECM	Extracellular matrix
EDTA	Ethylene diaminetetraacetic acid
EGF	Epidermal growth factor
ELISA	Enzyme-lined immunosorbant assay
EMT	Epithelial to mesenchymal transition
ER	Estrogen receptor
ERK	Extracellular signal-regulated kinase
FBS	Fetal bovine serum
FGF	Fibroblast growth factor
FSH	Follicle stimulating hormone
FSHR	Follicle stimulating hormone receptor
g	Acceleration of gravity
GAG	Glycosaminoglycan
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GC	Granulosa cell

GCNF	Germ cell nuclear factor
GDF	Growth differentiation factor
GDNF	Glial cell-derive neurotrophic factor
GS-domain	Glycine-serine rich domain
h	Hour
HAS	Hyaluronan synthase
HC	Heavy chain of IαI
hCG	Human chorionic gonadotropin
HHT	Hereditary hemorrhagic telangiectasia
IαI	Inter alpha inhibitor
ICSI	Intracytoplasmic sperm injection
ID	inhibitor of differentiation factor, inhibitor of DNA binding factor
IGF	Insulin-like growth factor
IL	Interleukin
I-SMAD	Inhibitory SMAD
IU	International unit
IVF	*In vitro* fertilization
IVM	*In vitro* maturation
JNK	c-Jun N-terminal kinase
Kb	Kilobase
kDa	KiloDaltons
KL	Kit ligand
LDL	Low-density lipoprotein

LH	Luteinizing hormone
LHR	Luteinizing hormone receptor
μ	Micro
MAPK	MAP kinases
MEKK	MAPK/ERK kinase kinase
MH	MAD-homology
ml	Milliliter
min	Minutes
MMP	Matrix metalloproteinase
mRNA	Messenger ribonucleic acid
MW	Molecular weight
n (as in nM)	Nano
NF-κB	Nuclear factor kappa B
OD	Ovarian dysgenesis
OSF	Oocyte-secreted factor
P450scc	P450 cholesterol side-chain cleavage enzyme
P450c17α	cytochrome P450 17α-hydroxylase/17,20-lyase
PA	Plasminogen activator
PAGE	Polyacrylamide gel electrophoresis
PBS	Phophate-buffered solution
PCOS	Polycystic ovary syndrome
PCR	Polymerase chain reaction
PCSK	Proprotein convertase subtilisin/kexin

PDGF	Platelet derived growth factor
PGC	Primordial germ cell
PGE	Prostaglandin
PK (as in PKA and PKC)	Protein Kinase
PI3K	Phosphatidylinositol-3-kinase
POF	Premature ovarian failure
PR	Progesterone receptor
PTX3	Pentraxin 3
RNA	Ribonucleic acid
R-SMAD	Receptor-activated SMAD
RT-PCR	Reverse transcription polymerase chain reaction
SBE	SMAD binding element
SD	Standard deviation
SDS	Sodium dodecyl sulphate
Sec	Seconds
Ser	Serine
SF-1	Steroidogenic factor 1
siRNA	Small interfering RNA
SMAD	Son of mothers against decapentaplegia
SP1	Specificity protein 1
StAR	Steroidogenic acute regulatory protein
STAT	Signal transducer and activator of transcription
TAB	TAK interacting protein

TAK	Mitogen-activated protein kinase kinase kinase 7
TEMED	N,N,N'N'-tetramethylethlenediamine
TGF	Transforming growth factor
TGFBR2	TGF type II receptor
Thr	Threonine
TNFα	Tumor necrosis factor α
Tris	Tris (hydroxyl methyl) aminomethane
TSG-6	TNF-stimulated gene 6 protein
TSP	Thrombospondin
YY1	Transcription factor Yin Yang 1
VEGF	Vascular endothelial growth factor

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Chapter 1: Introduction

1.1 Ovarian functions

1.1.1 Folliculogenesis

As early as the third week of gestation, the earliest primordial germ cells (PGCs), which originate from the extra-embryonic ectoderm, migrate to the primitive gonadal fold, and proliferate to a total number of 7 x 10^6 cells. This process requires bone morphogenetic protein (BMP) 4 and BMP signaling [1]. During embryonic life, PGCs, which are clustered in germ cell nests, undergo a continuous decrease to 1 x 10^6 cells [2, 3]. Around birth, the germ cell nests break down to allow the formation of primordial follicles, which are composed of one oocyte with a single layer of pregranulosa cells surround the oocyte [3]. The transition from a primordial to a primary follicle is characterized by the change of the granulosa cell layer from a squamous shape to a cuboidal shape, as well as increased oocyte diameter [3]. As primary follicle development progresses, theca layers appear, which come from the mesenchymal stroma cells, accompanied with proliferation of the granulosa cells [3-5]. At this stage of folliculogenesis, follicle stimulating hormone (FSH) is not involved in the proliferation of granulosa cells or theca cells. Growth factors are believed to induce theca cell recruitment and granulosa cell proliferation [6-8]. As primary follicles grow into preantral follicle, the zona pellucida, a thick layer of glycoprotein and proteoglycans produced by the granulosa cells, appears between the granulosa cells and the oocyte [9]. Under the stimulation of FSH, granulosa cells proliferate into multiple layers and are separated from the theca cell layers by a basement membrane called the basal lamina [10]. In antral follicles before ovulation, the multiple theca layers can be divided into two types: the theca interna, which is adjacent to basal lamina and the theca externa which is adjacent to stromal tissues [11].

In the first few days of every menstrual cycle, a small group of primordial follicles are recruited to respond to FSH and begin to mature [12, 13]. Only one of these follicles is selected to become the pre-ovulatory follicle in humans and undergo ovulation. The other follicles stop growing and become atretic follicles. This selection is mainly regulated by gonadotropins (FSH and luteinizing hormone (LH) [14-17] and is also mediated by locally synthesized growth factors such as Transforming Growth Factor (TGF-β) and Insulin-like Growth Factor (IGF) [18]. After ovulation, the enlarged granulosa cells, surrounding theca cells, surrounding stroma cells, endothelial cells, leukocytes and fibroblasts become the corpus luteum [19]. The steroidogenic luteal cells, which are the main source of ovarian steroid hormone production post ovulation, come from the granulosa cells and theca cells [20]. The non-steroidogenic cells occupy 70-80% of the corpora luteum and regulate corpora luteum functions by formation of neovascular networks [21]. During the whole reproductive life of a woman, only 300-450 follicles are ovulated from the initial count of 300,000 primordial follicles [22]. Apoptosis is very important in folliculogenesis by regulating puberty, atresia at menopause and corpus luteum regression [22].

1.1.2 Steroidogenesis

1.1.2.1 Estradiol synthesis

In the ovary, granulosa cells are the cellular source of estradiol and progesterone and the theca cells are the cellular source of androgens. In the antral follicles, estradiol production depends on both of these cell types (granulosa cells and theca cells) and is induced by two gonadotropins (FSH and LH) [23].

Critical to this two-cell, two-gonadotropin mechanism, LH receptors are expressed on theca cells and FSH receptors are expressed on granulosa cells of antral follicles. When LH binds

to receptors, theca cells uptake cholesterol, a substrate of steroid hormones. Cholesterol is mainly present in blood as low-density lipoprotein (LDL) and transferred into cells by binding LDL receptors [24]. Steroidogenic acute regulatory protein (StAR) governs the process by which cholesterol is transported to the inner mitochondrial membrane where the first reaction of steroidogenesis takes place by cytochrome P450 side-chain cleavage enzyme (P450scc)[24, 25]. Pregnenolone is produced in this reaction catalyzed by P450scc. Pregnenolone is converted to progesterone by 3β-hydroxysteroid dehydrogenase (3βHSD). Progesterone then subsequently cleaved by a unique enzyme in theca cells, cytochrome P450 17α-hydroxylase/17, 20-lyase (P450c17), to form androstenedione. Androstenedione can be reduced to testosterone by 17β-hydroxysteroid dehydrogenase (17βHSD). LH stimulates the expression of LDL receptors, StAR, P450scc and P450c17, and induces cholesterol to be taken up and induces androgen productions [26, 27].

Androgens produced by LH-stimulated theca cells diffuse into granulosa cells and become the main substrates for estrogen synthesis. Androgens are converted into estrogens (estrone and estradiol) by an aromatization reaction that is catalyzed by cytochrome P450 aromatase, which is the enzyme exclusively expressed in granulosa cells [28]. FSH increases the expression of aromatase, as well as LH receptors in granulosa cells before an LH surge. Estradiol is the main steroid hormone produced by ovaries during folliculogenesis until the LH surge occurs, which leads to StAR and LDL receptor expression in granulosa cells. This leads to granulosa cells producing progesterone and the start of luteinization.

1.1.2.2 Progesterone synthesis

Luteinization is the process when granulosa cells and theca cells differentiate into luteal cells, and is regulated by LH. The main function of luteal cells is progesterone secretion to maintain pregnancy or regulate the estrus cycle. After ovulation, increasing theca cell vascularization brings more cholesterol to luteinized granulosa cells and a high level of LH induces LDL, StAR, P450scc and 3βHSD expression. This results in a large amount of progesterone production [29-32]. Pregnenolone is produced in granulosa cells and is then converted into progesterone by 3βHSD [33]. During this process, growth factors from oocytes, theca cells and granulosa cells play an important role in regulation of progesterone production regulation and luteinization in a paracrine or autocrine manner.

The rate-limiting step of steroidogenesis is the transport of cholesterol from the outer to the inner mitochondrial membrane in steroid hormone-making cells in the adrenal gland, ovary and testis [34]. This step is controlled by StAR [34]. StAR is synthesized as a 37 kDa precursor protein. After translocation into the mitochondria, the specific mitochondrial leader sequence of StAR is cleaved to form a 30 kDa mature protein [35]. The expression of StAR is rapidly increased by intracellular cyclic adenosine monophosphate (cAMP), which is induced by gonadotropin hormones (LH, FSH and human chorionic gonadotropin (hCG) [36]. StAR expression is up-regulated or down-regulated by growth factors, such as insulin, IGFs [37, 38], TGF-β family members [39, 40], and Epidermal Growth Factor (EGF) [37]. Numerous transcription factors were reported to bind to the StAR promoter and regulate its activation. Steroid factor-1 (SF-1) controls the basal- and cAMP-induced transcription of StAR by binding to three different binding sites, and overexpression of cAMP-response elements-binding protein (CREB) increased cAMP-induced murine StAR promoter activation [41, 42]. The StAR

promoter is also stimulated by GATA4, GATA6, sp1/3 and CCAAT/enhancer binding protein (C/EBP) and suppressed by DAX-1 and transcription factor Yin Yang 1 (YY-1) [43].

1.1.3 Ovulation and luteinization

The release of an oocyte from a preovulatory follicle is a key event of the ovary. Following ovulation, the granulosa cells and theca cells left in the follicle undergo luteinization and form a transient endocrine gland called the corpus luteum. The complex process of ovulation and luteinization is composed of several morphological changes occurring sequentially and is closely regulated by many factors. Those changes can be divided into early events and late events. The early events start in the preovulatory follicles including cumulus-oocyte complex (COC) mass production and expansion, resumption of oocyte meiosis, reduction of cell-cell communication, and basement membrane breakdown. The late events start after the oocyte release (follicle rupture) and include luteinization (Figure 1-2). The successful ovulation/luteinization is governed by gonadotropins and regulated by steroid hormones and growth factors secreted by ovarian cells in the follicles [44] (Figure 1-3).

1.2 Periovulatory interval

1.2.1 Morphology changes during the periovulatory interval

1.2.1.1 COC expansion

As follicle growth progresses, the increasing follicular fluid of the antrum segregates granulosa cells into two subgroups: cumulus cells which are closely adjacent to the oocyte and mural granulosa cells which are layered against the follicle wall and close to the basement

membrane. The oocyte and the surrounding cumulus cells and the viscoelastic extracellular matrix constitute the unique hyaluronan-rich COC matrix [45].

Hyaluronan, a ubiquitous extracellular matrix (ECM) component, composes the backbone of the COC matrix in vertebrates and is essential for successful ovulation [46]. Three types of hyaluronan synthases (HASs) are expressed in mammalian cells to catalyze the hyaluronan biosynthetic reaction: HAS1, HAS2, and HAS3 [47]. HAS2, which synthesizes an extremely large hyaluronan, is the most important HAS during COC expansion [48]. The role of hyaluronan is to fill the intercellular spaces of the COC matrix which is stabilized by numerous hyaluronan binding proteins such as tumor necrosis factor stimulator gene-6 (TSG-6), inter-α trypsin inhibitor (IαI) and versican (Figure 1-1). In the presence of a converting enzyme, the heavy chains (HCs) of IαI and TSG-6 become directly linked to hyaluronan forming the base of the complex matrix [49-52]. There are two types of fundamental tertiary hyaluronan-binding complexes: hyaluronan-IαI HC-IαI HC and hyaluronan-TSG-6-IαI HC [53, 54]. Pentraxin 3 (PTX3), which does not have a hyaluronan-binding motif, binds to the TSG-6 of the tertiary hyaluronan-binding complex: hyaluronan-TSG-6-IαI HC, and regulates COC mass density [53, 54]. Another hyaluronan-binding protein that may stabilize the COC matrix is versican which is also found in a large number of tissues. Versican is produced by granulosa cells and then localized in the expanded cumulus matrix, basal lamina and follicular fluid [55]. The proteolytic cleavage of versican by A Disintegrin and Metalloproteinase with TromboSPondin Repeats-1 (ADAMTS-1) might be involved in COC expansion and ovulation [56] (Figure 1-1). The synthesis of hyaluronan and hyaluronan interacting proteins are regulated by gonadotropins and oocyte-secreted factors.

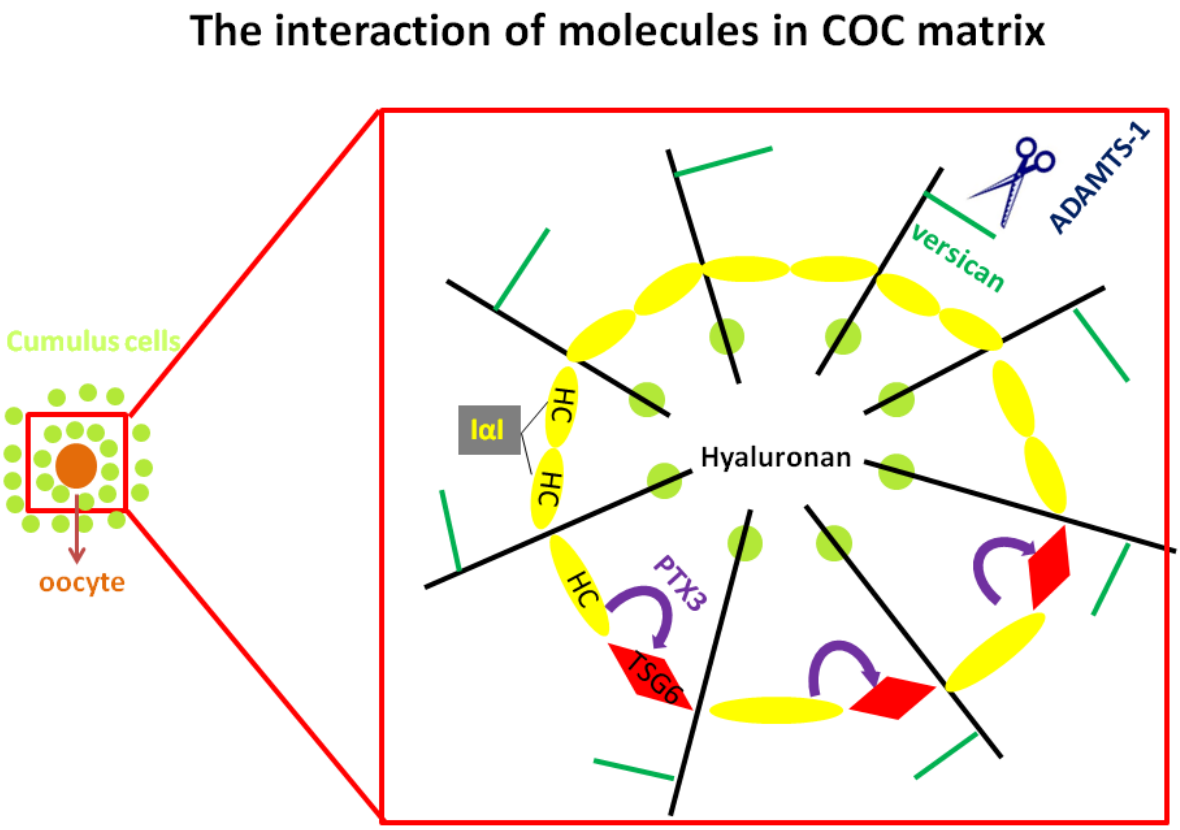


Figure 1-1 The interaction of molecules in COC matrix.

There are two types of fundamental tertiary hyaluronan-binding complex: hyaluronan-IαI HC-IαI HC and hyaluronan-TSG-6-IαI HC. PTX3 can bind to the TSG-6 of the hyaluronan-TSG-6-IαI HC tertiary hyaluronan-binding complex. This will only occur if PTX3 previously encounters IαI. Versican, a proteoglycan, binds to hyaluronan directly and is proteolytically cleaved by ADAMTS-1.

1.2.1.2 Reduction of cell-cell communication and resumption of oocyte meiosis

Oocyte meiosis begins in embryonic PGCs and then arrests in the late diplotene stage until ovulation. Cyclic guanosine monophosphate (cGMP), which diffuses into oocytes from granulosa cells through gap junctions, maintains the inhibitory effects on meiosis [57]. At the time of ovulation, oocytes restart meiosis after withdrawing meiosis inhibitory factors because of the reduction of cell-cell communication. The LH surge decreases connexin 43, the primary connexin that comprises the gap junctions in follicles [58] and is involved with junction permeability [59], and decreases cGMP levels in the COCs [60]. The reduction of gap junctions between granulosa cells and cumulus cells results in the decrease of cGMP signaling and oocyte meiosis resumption [57, 58, 60].

1.2.1.3 Basement membrane breakdown, follicle rupture and luteinization

During the periovulatory interval, the basement membrane between granulosa cells and theca cells degrades and endothelial cells, luteinizing theca cells and fibroblasts migrate into the luteinizing granulosa cells. During these processes, as well as the remodeling and turnover of the ECM, are controlled by the action of proteinases, such as plasminogen activators (PAs) and matrix metalloproteinases (MMPs), that cleave type IV collagens and leads to ECM degradation and basement membrane breakdown [61-64]. Oocyte rupture is the result of proteolytic digestion of the follicular apex, which is also called the stigma. A critical proteinase, ADAMTS-1, is secreted by mural granulosa cells and is believed to contribute to oocyte rupture by cleaving versican and aggrecan located in the basement membrane [56].

The breakdown of the basement membrane is also essential for initiating luteal angiogenesis by allowing endothelial cell motility and migration and the spread of angiogenic

factors from granulosa cells [65]. This process results in a corpus hemorrhagicum which releases a large amount of progesterone into peripheral circulation.

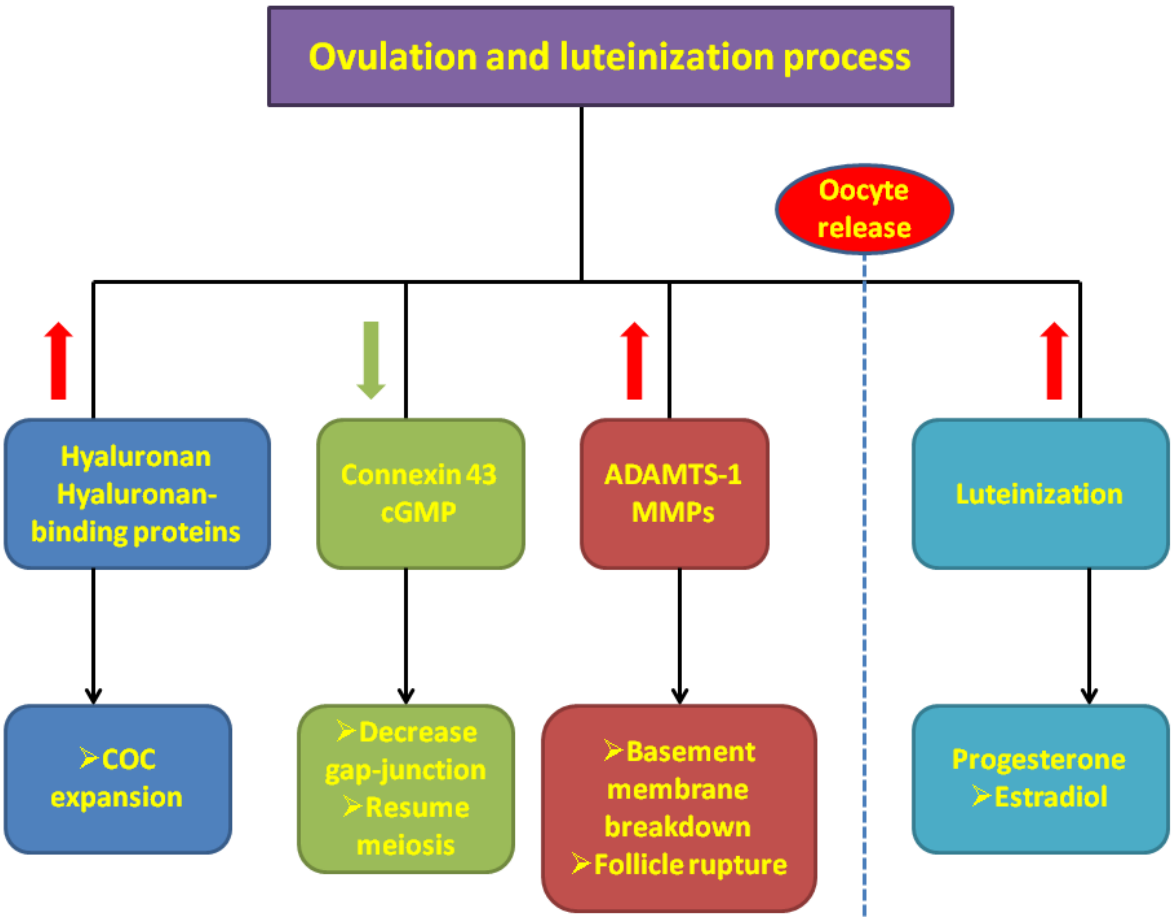


Figure 1-2 Morphological changes during the periovulatory interval

The complex process of ovulation and luteinization is composed of several morphological changes occurring with appropriate timing which are closely regulated by many factors. Those changes can be divided into early events and late events. The early events start in the preovulatory follicles, and include the hyaluronan-rich COC matrix formation and expansion, reduction of cell-cell communication accompanied with decreased connexin43 and cGMP. This results in oocyte meiosis resumption. The late events start after the oocyte release (follicle rupture) including luteinization.

1.2.2 The molecular regulators during the periovulatory interval

1.2.2.1 Gonadotropins and EGF-like growth factors

The LH surge, which occurs 34 to 36 hours prior to ovulation, triggers intracellular signaling activation and ovulation-related gene transcription to support the morphologic changes during ovulation and luteinization including the COC expansion, cell-cell gap junction decrease, basement membrane breakdown, follicle rupture, the secretion of prostaglandins and steroid hormones, and luteal angiogenesis [66] .

Before ovulation, FSH stimulates the expression of LH receptors in mural granulosa cells. By contrast, in cumulus granulosa cells, expression of the LH receptors is inhibited by the oocyte [67], but the FSH receptors are still expressed [68]. The synthesis of hyaluronan rapidly increases with FSH treatment. Otherwise, without FSH, there is very little COC mass (only 10% in the absence of FSH) [69] in *in vitro* COC cultures. Since there are fewer LH receptors expressed in cumulus cells compared to mural granulosa cells [70], LH evidently promotes the COC matrix formation by stimulating expression of the epidermal growth factor (EGF)–like growth factors, such as amphiregulin, betacellulin and epiregulin, and triggering EGF signaling. This activation of EGF signaling stimulates expression of COC expansion-related genes [71, 72].

The LH surge results in the reduction of the cell-cell gap junction by decreasing connexin 43 expression [59] and the breakdown of basement membrane by inducing both ECM components (e.g. versican) [73] and production of a series proteinases (e.g. ADAMTS-1) [74].

1.2.2.2 Steroid hormones

Multiple studies have suggested that progesterone [75, 76] and estradiol [77] work as local mediators in ovulation. Two isoforms of progesterone receptors (PRs), which are expressed

in the ovaries of human and other species, are the full-length PRB and an N-terminal truncated PRA which lacks 164 amino acids [78-80]. In human ovaries, PRs are detected in granulosa cells of dominant follicles after the LH surge and in stromal cells throughout the menstrual cycle [81]. They also persist in luteinized granulosa cells and in early corpus luteum. Similar results are confirmed in other species such as monkey [82], rabbit [83], rat [84, 85], and chicken [86].

In mice and rats, ovulation and follicle rupture can be inhibited by administration of PR antagonists such as RU486 [87-89], ZK28299 [90], and CDB-2914 [91, 92]. Women administered RU486 exhibited impaired ovulation [93]. Women administered with 100 mg CDB-2914 experienced delayed or blocked ovulation and an increased incidence of luteinized, unruptured follicles [94]. In female *Pgr* null mouse models, no changes on size, weight and overall external appearance of ovaries were found compared to wild-type mice. However, histological studies revealed that there was complete anovulation with unruptured follicles after gonadotropin stimulation. Interestingly, granulosa cells in those unruptured follicles exerted normal cumulus expansion, normal LH-induced Cyclooxygenase-2 (COX2) expression and normal expression of P450scc [95-97].

The major defect in estradiol receptor (ER) knockout mice is a disruption in negative feedback at neuroendocrine centers resulting in high levels of FSH receptors and LH receptors [98]. In mouse ovaries, ER-β expression was found in granulosa cells and ER-α was expressed in thecal and interstitial cells [99]. This suggests that ER-β is the main receptor involved in estradiol-regulated granulosa cell growth. Indeed, mice deficient in ER-β have been shown to have reduced cumulus cell mass and have a decreased rate of ovulation [100]. Additionally, mouse *in vitro* COC cultures have a decreased expansion index as well as decreased *Has2* mRNA transcripts (but neither *Ptx3* nor *Tsg6*). These deficiencies could be reversed by

exogenous estradiol supplementation. Moreover, the same study also revealed that GDF9 and BMP15 cooperated with estradiol to promote COC expansion [77].

In summary, these studies indicated that PR is required for preovulatory follicle rupture and oocyte release [95, 97] while estradiol and ER-β are required for COC mass production and COC expansion [77, 100].

1.2.2.3 Prostaglandins and COX2

The production of prostaglandin E_2 and $F_{2\alpha}$ ($PGE_2$and $PGF_{2\alpha}$, respectively), primarily PGE_2, synthesized by COX2, is essential for COC expansion, and oocyte rupture. Functioning not only as a proteolytic enzyme within the follicular wall, PGE_2 also triggers an inflammatory-like response during ovulation and luteinization [101-103]. Studies using a pharmacological inhibitor of COX2 indicated that follicle rupture is blocked without affecting oocyte maturation and luteinization in both human and animals [104, 105]. In human granulosa cells, both PGE_2 and $PGF_{2\alpha}$ are stimulated by interleukin-1β which is an inflammatory response [106]. PGE_2 and COX2 are also stimulated by LH-induced EGF-like signaling and transforming growth factor-β (TGF-β)-induced SMAD2/SMAD3 signaling [107, 108]. In the mouse, *Cox2* expression in granulosa cells is stimulated by oocyte secreted factors (OSFs), such as growth differentiation factor (GDF)9 and BMP15, which may explain the function of OSFs on ovulation [109, 110]. The functions of PGE_2 are well studied in ovulation. In the clinical setting, infertility patients are advised to avoid using drugs which may inhibit PGE_2 synthesis [111].

1.2.2.4 Oocytes and OSFs

It has been widely believed that oocytes exert important roles to regulate granulosa cells functions. Briefly, during the ovulation/ luteinization process, oocytes have two main functions to regulate granulosa cells through OSFs. First, the separation of the oocyte from COC results in a spontaneous luteinization in cumulus granulosa cells. Moreover, co-culture mural granulosa cells with an isolated oocyte decreased FSH-induced expression of LH receptors [67]. These studies indicate that oocytes with OSFs have the ability to inhibit luteinization.

Second, when oocytes were separated with cumulus cells in COCs, cumulus cells stopped expansion. This impaired expansion could be rescued by adding back culture medium with OSFs. This result indicates that OSFs have the ability to induce COC expansion [112, 113]. The half-life of HAS2 is very short in granulosa cells based on *in vivo* and *in vitro* studies. The synthesis of hyaluronan is rapidly increased by FSH treatment with the maximum rate lasting between 4 and 10 hours but then synthesis declines by 18 hours post-treatment. Moreover, when the transcription of HAS2 mRNA is inhibited, the maximum rate can only be sustained for 2 hours. The synthesis of hyaluronan was minimal after FSH treatment when the oocytes were removed from COCs. This inhibition was reversed by the oocyte or when the conditional medium with OSFs was added back to the culture. This suggests that the maximal synthesis of hyaluronan requires two distinct factors: FSH and OSFs [48, 113-115].

1.2.2.5 TGF-β superfamily members

As prime candidates of OSFs, the functions of TGF-β superfamily members during the process of ovulation are correlated with the roles are that the oocyte has: inhibiting luteinization and promoting COC expansion. In human granulosa cells, GDF9 inhibits 8-Br-cAMP-stimulated

progesterone [116] and TGF-β [117] and BMP15 [118] decrease basal progesterone. In animal models, SMAD2/3 signaling, induced by GDF9 and TGF-β, is essential to maintain COC expansion [119]. Moreover, the COX2 expression and PGE2 production, which are essential for COC expansion and subsequent ovulation, are induced by TGF-β in human [108] and GDF9 [109], as well as BMP15 [110] in mouse granulosa cells. In human cumulus cells from patients undergoing intracytoplasmic sperm injection (ICSI), the mRNA levels of GDF9 and BMP15 were closely associated with oocyte maturation, fertilization and embryo quality [120]. In summary, TGF- β superfamily members are essential to regulating the timing of the ovulation/luteinization process by inhibiting luteinization, assisting COC expansion and regulating other ovulation events.

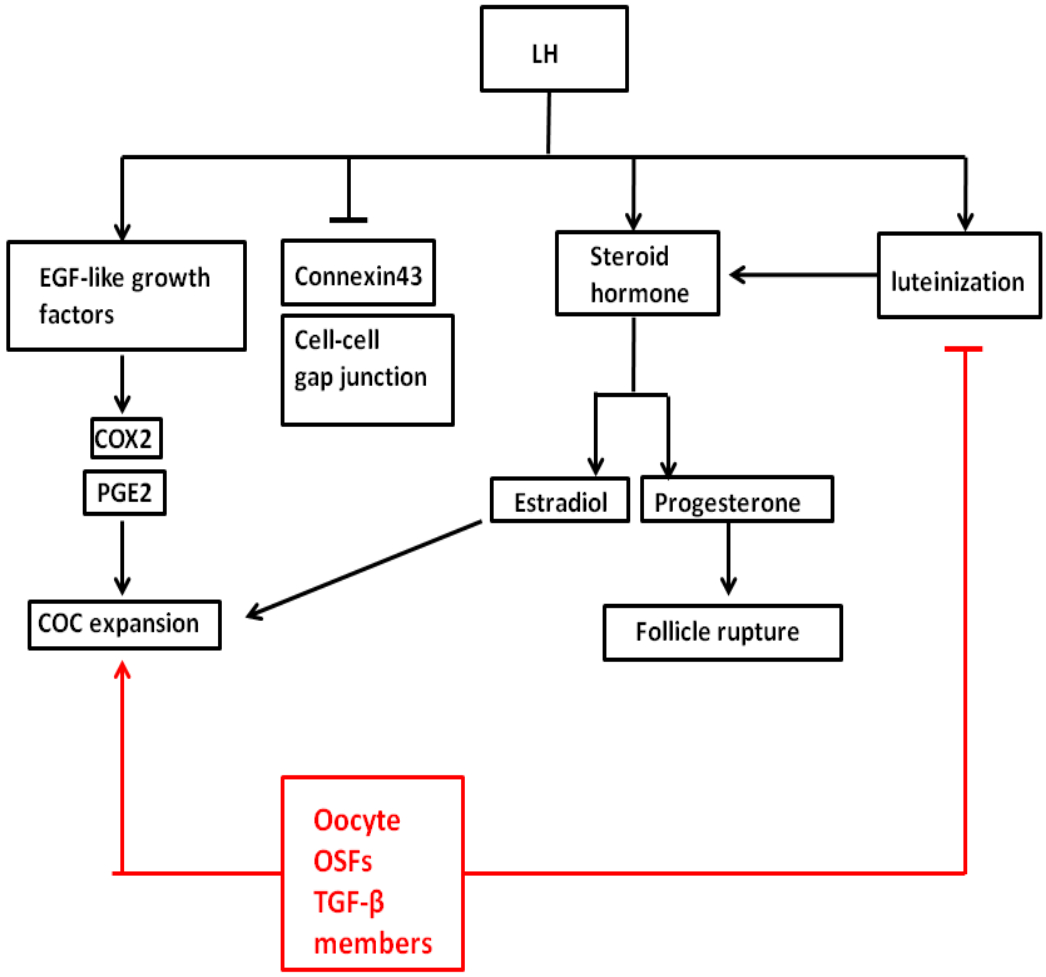


Figure 1-3 The molecular regulators during the periovulatory interval

The LH surge induces both ovulation and luteinization. LH-induces EGF-like growth factors (amphiregulin, betacellulin and epiregulin), and triggers EGF signaling pathway, which induce COX2 expression and PGE_2 production and COC expansion. The LH surge results in reduction of cell-cell gap junction by decreasing connexin 43 expressions, and induces estradiol production and progesterone production. Estradiol and ER- β is required for the COC matrix formation and expansion, while PR is required for preovulatory follicle rupture and oocyte release. The steroid hormones and growth factors secreted by ovarian cells play critical roles in appropriate timing of ovulation/luteinization process. During the periovulatory interval, oocytes exert two main functions to regulate granulosa cells through OSFs (mostly TGF-β superfamily members): promoting COC expansion and inhibiting granulosa cells luteinization.

1.3 Bone morphogenetic proteins (BMPs)

1.3.1 BMPs belong to TGF-β superfamily

The TGF-β superfamily of proteins includes more than 40 ligands that can be divided into several groups: the TGF-β group including TGF-β1, 2, 3, the activin/inhibin group including activin A, AB, B, C, D, E and inhibin A, B, the BMP group including more than 20 BMPs, the GDF group including 9 members, the glial cell-derive neurotrophic factor (GDNF) group including GDNF, artemin and neuturin and other members including nodal, lefty and anti-müllerian hormone (AMH) [6].

All members of this superfamily share a similar structure consisting of three parts: a signal peptide, a prodomain and a mature peptide. The signal peptide directs these ligands to be properly secreted out of the cells where they bind to TGF-β receptors (type I and type II) and carry out multiple functions related to cell homeostasis. TGF-β ligands share a conserved structure: cystine knots formed by cystine residues. There are six to nine cystine residues located on the prodomain and the mature peptide of TGF-β ligands depending on the different subgroups of TGF-β members. These cysteine knots play an important role for TGF-β dimerizing and functioning as homodimers or heterodimers [121]. However, some TGF-β members do not form covalent dimers by cysteine knot like BMP15 and GDF9. TGF-β members have a wide array of functions in the body by regulating cell proliferation/apoptosis, differentiation, migration, invasion and extracellular matrix production which impact development, normal physiology and disease processes [122].

1.3.2 Structure of BMPs

In the 1960's, BMPs were identified to induce ectopic bone formation [123]. Later on, many studies demonstrated that BMPs have the ability to induce bone and cartilage formation from mesenchymal stem cells, to induce embryogenesis and development and to regulate the maintenance of adult tissue homeostasis.

Similar to other TGF-β superfamily members, BMPs are comprised of three parts: a signal peptide, an N-terminal prodomain for folding and secretion, and a C-terminal mature domain [124]. BMPs are secreted from cells, with predomains, as latent homodimer or heterodimer complexes, such as BMP15/GDF9 and BMP2/BMP7 [125-127]. The BMP precursors are cleaved by a proprotein convertase family composed of nine members called proprotein convertase subtilisin/kexin (PCSK1-PCSK9). One example is PCSK3 (furin) located at the linker site (RXXR) between the prodomain and the mature domain [124]. The mature BMPs contain seven cysteine residues: six of them can form intramolecular disulfide bonds known as cysteine knots and the seventh cysteine is used for dimerization with another monomer via a disulfide bond [128]. BMP15 is also known as GDF9B because of the homologous identity, expression and function compared to GDF9 [129]. Both BMP15 and GDF9 lack the seventh cysteine for dimer formation. The structure of a monomeric BMP ligand has a "left hand" shape to the protein folding pattern including a "central α-helix-wrist" and "two pairs of antiparallel β-sheets fingers". The dimeric BMPs have four hydrophobic patches as a functional motif: two "knuckle" epitopes bind to two type II TGF-β receptors and two "wrist" epitopes bind to two type I receptors. The N-terminal segments bind to extracellular matrix proteins, including fibrillin and heparin, to immobilize BMPs at the extracellular cell surface [128].

1.3.3 Signaling pathway of BMPs

1.3.3.1 SMAD-dependent signaling

BMPs initiate their cellular signaling cascade by binding to two cell-surface receptors: TGF-β type I and type II. Both types of receptors are serine/threonine kinase receptors and consist of three parts: an extracellular domain, a transmembrane domain and an intracellular kinase domain [130]. TGF-β family members have seven type I receptors (ALK1-7). Four of these type I receptors bind with BMP ligands: ALK2 (ActR-1A), ALK3 (BMPR-1A), ALK6 (BMPR-1B) and ALK1, which is specific for endothelial cells [131, 132]. There are five type II receptors which bind with TGF-β ligands: ActR2, ActR2B, AMHR2, TGFBR2 and BMPR2. Three of these five type II receptors, ActR2, ActR2B and BMPR2, are also known to interact with BMPs. Some of TGF-β members, such as TGF-β1 and activin, first bind to the type II receptors with continuous activated kinase activity. Then, the TGF-β type I receptors are recruited into the complex and phosphorylated by TGF-β type II receptor kinases a region rich of glycine and serine residues (GS domain). The GS domain is exclusive to type I receptors. The third receptor type, such as betaglycan, may be necessary for enhancing binding affinity for some TGF-β ligands, including inhibin [133]. However, BMPs have low binding affinity to both type I and type II receptors individually, and bind to both types of receptors equally [134-137].

After TGF-β type I receptor phosphorylation, a conformational change occurs at the GS domain that allows ATP to bind and activates a downstream signaling cascade beginning with the receptor-activated SMAD (R-SMAD) [138]. Besides R-SMAD, which has two groups, SMAD2/3 and SMAD1/5/8, the SMAD family also includes the common-mediator SMAD (SMAD4) and inhibitory SMADs (SMAD6 andSMAD7). All the SMAD family members have two Mad-homology domains, MH1 and MH2, with a proline-rich linker region between them. R-

SMADs have a serine-rich motif, Ser-Ser-X-Ser (SSXS), at the C-terminal end which can be phosphorylated by TGF-β type I receptors [130]. BMP type I receptors activate canonical SMAD1/5/8 group which then associate with SMAD4, and this complex translocates into the nucleus to exert functions that regulate gene expression. SMAD6 and SMAD7 (inhibitory SMADs) regulate BMP signaling by a negative feedback loop [139]. In recent studies, BMPs were reported to activate noncanonical SMAD2/3 signaling, which is commonly induced by TGF-β, activin and GDF subgroup ligands, and to regulate cancer progression and hormone production [140, 141].

1.3.3.2 SMAD-independent signaling

Similar to TGF-β, BMPs were found to induce several SMAD-independent pathways in specific tissues. Mitogen-activated protein kinase (MAPK) signal pathway is believed to be the major SMAD-independent pathway induced by BMPs. BMP2 and BMP4 can induce TGF-β activated kinase 1 (TAK1) and bind with TAK binding protein 1(TAB1). This binding will induce apoptosis via downstream molecules, including p38 MAPK, C-jun N-terminal kinase (JNK) and nuclear factor kappa beta (NFkB) [142]. BMPs are also able to activate extracellular signal-regulated kinase (ERK), phosphoinositide 3-kinase (PI3Kinase), Protein Kinase (PK) A, PKC and PKD (PKCμ) [142-144].

1.3.4 The biological functions of BMP signaling

1.3.4.1 BMP signaling in embryogenesis:

BMP signaling is essential for embryogenesis because *Bmp2, Bmp4, Smad1, Smad5, Smad4, Alk2* or *Alk3* knockout mice all die at the embryonic age. Mesoderm formation and

cardiac development are most affected by BMPs during embryogenesis according to *Bmp* knockout mice models. There is no mesoderm formation in B*mp4* or *Alk3* knockouts [145, 146] and there are severe defects in cardiovascular development in *Bmp2, Bmp4, Bmp10, Smad4, Alk3, Alk2* or *Bmpr2* knockouts [147-155]. Also, BMP signaling is necessary for germ cell formation during embryogenesis because there is no PGC or defective PGC formation in mice deficient in BMP4, BMP8, SMAD1, SMAD5, or ALK2 [1, 150, 156-160]. BMP7- or BMP11-deficient mice die after birth with defects of the kidney and eye development [161-164].

1.3.4.2 BMP signaling in the skeletal system:

BMPs were initially identified as unknown factors which induce ectopic bone growth in muscle, and subsequently named "bone morphogenetic protein" [123]. By using knockout phenotypes of BMP signaling, most BMPs were shown to regulate the formation and homeostasis of bones, joints and cartilage. Deleting *Bmp2* results in spontaneous fractures and impaired fracture repair on limbs [165] and severe chondrodysplasia on chondrocytes [166]. BMP4 regulates digit patterning on limb bud mesoderm [167]. Mice deficient in BMP5 and BMP6 have smaller and weaker bones [168, 169], while *Bmp7* knockout mice exhibit defects in skeletal patterning but no defects involving limbs [170, 171]. The mice with deficiency of SMAD1 exhibited delayed calvarial bone development [172]. Mice with deletions of SMAD4 have lower bone mass and inner ear malformation because of chondrocyte development defects [173, 174].

Even though most BMPs have positive effects on skeletal formation, some BMPs are also reported as negative regulators in the skeletal system. BMP3 has been shown to decrease bone density [175] while *Bmp13* knockout mice have accelerated coronal suture fusion [176]. Deletion

of ALK3 in osteoblasts also induced bone mass [177]. The effects of BMPs on the skeletal system and disease are well studied and recombinant human BMP2 and BMP7 are currently used for spinal fusion, fracture healing and dental tissue engineering [178-180].

1.3.4.3 BMP signaling in cardiovascular and pulmonary systems:

The cardiovascular system is another system in which BMP signaling plays an important developmental role. Since *Bmp2* or *Bmp4* mutant organisms were embryonically lethal with severe cardiac development malformations including cardiac septation defects and valvular anomalies, demonstrating a role for BMPs in cardiovascular organogenesis in the embryo. BMP2 signaling has been shown to be essential for endocardial cushion (EC) formation by regulating expansion of ECM and the epithelial-to-mesenchymal transformation (EMT) [149, 153, 181, 182]. Similar to BMP2, BMP4 is required for atrioventricular (AV) septation formation [183]. Organisms with a double mutation of *Bmp6* and *Bmp7*, which are expressed in the EC, resulted in defective EC formation but there were no cardiogenesis defects by deleting either of them [184]. Besides BMPs, mutations of *Alk2, Alk3* and *Bmpr2,* resulted in defects of the AV septa, valves and EC formation [147, 153, 185, 186].

The role of BMPs in angiogenesis has been established from knockout animal models and human vascular diseases. BMP signaling deficiency (SMAD8 mutation and BMPR2 heterozygous mutations) were reported in pulmonary arterial hypertension (PAH) patients [187-189]. Hereditary hemorrhagic telangiectasia (HHT), characterized by fragile blood vessel formation in skin and mucosa, is another vascular disease that was found to be associated with BMP signaling, including ALK1 and SMAD4 [190-192].

In the pulmonary system, BMP4 was shown to induce lung development. Knocking out *Bmp4, Smad1,* or *Alk3* in lung epithelium resulted in abnormal branching morphogenesis and airway formation that caused severe neonatal respiratory failure [193-195].

1.3.4.4 BMP signaling in the urinary system

BMPs have been shown to regulate urinary system development during embryogenesis and have a protective role against renal disease by regulating adult renal homeostasis. As mentioned previously, *Bmp7* or *Bmp11* knockout mice die after birth with severe kidney defects. *Bmp11* knockout mice have a failure of ureteric bud formation [163], and *Bmp7* knockout mice have renal dysplasia because of an interruption in growth and development [161]. BMP7 has also been shown to protect from chronic kidney disease. Exogenous administration or transgenic overexpression of BMP7 reduced renal fibrogenesis and apoptosis as well as transdifferentiation of epithelial cells[196].

1.3.4.5 BMP signaling in the reproductive system

Several BMPs have been shown to be essential in reproductive system development and biology. As mentioned previously, BMP signaling is necessary for germ cell formation during embryogenesis because there is no PGC or defective PGC formation in mice deficient in BMP4, BMP8, SMAD1, SMAD5 or ALK2 [1, 150, 156-160]. In the male reproductive system, BMP8 is believed to play an important role in spermatogenesis and epididymis development [157, 197, 198]. The knockout mice of S*mad4* exhibit testicular dysgenesis [199]. *Alk3* knockout mice had abnormal Leydig cells [200].

In the female reproductive system, BMP15, also named as GDF9B, is the most well-studied BMP in mammal ovaries [201], based on its several features related to ovarian functions. First, BMP15 is exclusively expressed in oocytes of the rat [202], human and other mammalian oocytes [203-206]. Second, BMP15 has a homologous structure with another oocyte-derived TGF-β family member, GDF9, and can directly interact with GDF9 as heterodimers to exert functions in the ovaries [201, 207, 208]. Third, the naturally-occurring mutant phenotype in sheep and human and *in vivo* experiments on mice indicate BMP15 is essential for normal ovarian function.

Using genetic linkage, *BMP15* was shown to be the candidate gene for Inverdale sheep ($FecX^{I}$) and Hanna sheep ($FecX^{H}$) [203]. Those sheep carry a naturally-occurring X-linked mutation: in which sheep homozygous for the mutation are infertile (streak ovaries and a block in folliculogenesis at the primary stage) and those with a heterozygous genotype have increased ovulation (increased ovulation quota and litter size) [209, 210]. Since then, *BMP15* mutant sheep with similar phenotypes to Inverdale and Hanna were reported. Cambridge and Beclare sheep ($FecG^{H}$) breeds exhibited extreme variation in fertility from superovulation to normal ovulation to infertility. The *GDF9* gene mutation is associated with a sterility phenotype. The *BMP15* polymorphism ($FecX^{G}$ and $FecX^{B}$) and the *BMP15/GDF9* double heterozygote sheep were also similar in the ovulation rate of these two types of sheep [211]. Similarly, Rasa Araonesa ($FecX^{R}$) sheep and Lacaune sheep ($FecX^{L}$), new *BMP15* mutants, also displayed with increased ovulation in heterozygotes and sterility in the mutant homozygotes [212-214]. The function of BMP15 on reproduction in sheep was also studied by immunizing wild-type sheep against the active BMP15 protein. Similar to the phenotype in the natural mutant sheep, the short term immunization against BMP15 resulted in an increase in ovulation. Prolonged dosing at higher concentrations of

the immunization resulted in anovulation in the sheep [215, 216]. In both sheep and cattle, the antibodies against the BMP15 peptide resulted in anovulatory phenotypes which indicate that BMP15 is a key protein for ovulation rate in ruminant ovaries [217, 218].

Mutations of *BMP15* in human female patients with premature ovarian failure (POF) indicate clinical significance of the functions of BMPs on the reproductive system [219, 220]. The first report of BMP15 related to premature ovarian failure consisted of two sisters who carried an A-G transition of the *BMP15* gene (at position 704) and had ovarian dysgenesis (OD). They were heterozygous carriers but had similar phenotypes as to the homozygous mutant BMP15 sheep (streak ovaries with reduced granulosa cell proliferation) [220]. This indicated that BMP15 affected infertility by affecting both folliculogenesis and ovulation in the mammalian female. A number of other *BMP15* mutations were reported in POF patients with two representative mutants that occurred at a high incidence: $BMP15^{R76C}$ and $BMP15^{R206H}$. These mutations were not identified in normal women. These mutations were located in the region of the *BMP15* gene which translated the prodomain of the BMP15 protein. The impaired translation of BMP15 prodomain affected posttranslational processing of BMP15 which resulted in a reduced production of the mature BMP15 [221-223]. The relationship between POF patients and the *BMP15* mutation indicated that BMP15 is critical for female folliculogenesis and ovarian reserve in human beings.

BMP15 is involved in folliculogenesis and ovulation. *In vivo*, *Bmp15* null female rats were subfertile with follicular-defected development and decreased ovulation because more follicles had trapped oocytes within the follicles. Heterozygous rats were hardly affected compared to the wild type rat [201]. Similar to *Bmp15* null female rats, more severe fertility defects were found for *Bmp15*/*Gdf9* double mutant mice, which showed reduced fertilization, the

failure of adhesion between cumulus cells and oocytes, the absence of cumulus expansion and the existence of large oocytes [201]. *In vitro*, BMP15 treatment stimulated granulosa cells proliferation and mitosis in mice [224] and human [225], and induced COC expansion in mouse [110], cow [226] and pig [206].

Other BMPs have also been reported to have similar functions as BMP15 on ovulation and folliculogenesis. A study using recombinant human BMP6 infusion into the ovarian artery in sheep ovaries at both the early follicular phase and the luteal regression phase indicated that BMP6 can induce an acute stimulation of inhibin A, androstenedione and estradiol production in early follicular phase. This resulted in a precocious LH surge, ovulatory follicle maturation and formation of smaller corpora lutea in smaller-size follicles. However, the ovulation rate, the LH-induced estradiol, androstenedione or inhibin A were not significantly different between the control and the BMP6-infusion groups [227]. Some studies on BMP6 knockout mice have been inconsistent. One study using *Bmp6* null mice did not show a significant reduction in female fertility [228], while another reported that *Bmp6* knockout female mice exhibited a reduced ovulation rate, decreased response to gonadotropins and decreased transcripts of COC expansion-related genes induced by LH/hCG [229]. However, there was no effect of BMP6 on follicular development or LH receptor expression in the same study [229].

BMP2, BMP4 and BMP7 are mostly studied in follicles development, since the knockout mice of these BMPs die during embryogenesis [145, 161], or after birth [161]. BMP4 is required for PGC formation in mice [230]. In human fetal ovaries, BMP4 expression decreased after 8-9 gestational weeks. BMP4 promoted primordial granulosa cell apoptosis in the first trimester human fetal ovary (10 days) and elevated caspase-3 and increased MSX2 [231]. As early as the primordial follicle phase, BMP4 and BMP7 were found to stimulate primordial follicle growth

and to induce primordial to primary follicle transition in rat ovaries [6]. Treatment with BMP4 on neonatal rat ovaries increased the proportion of developing primary follicles. Treatment with BMP4 neutralizing antibody increased the progressive loss of primordial follicles and cellular apoptosis [232]. Similarly, a study injected BMP7 into rat left ovarian bursa with the right one as the control. They found that BMP7 stimulated primordial follicle growth and induced primordial to primary follicle transition in early follicle stage, and increased DNA synthesis and proliferation of granulosa cells in the late antral follicle stage [233]. However, neither BMP4 nor BMP7 were found to affect cellular proliferation/survival in sheep [234]. In cow granulosa cells, BMP4 and BMP7 suppressed apoptosis by stimulating survivin and XIAP mRNA. BMP7 also decreased caspase-9 [235]. Interestingly, BMP2 has been shown highly expressed in atresia and luteolysis granulosa cells but not expressed in predominant follicles and in the healthy corpus luteum in rat and sheep [234]. This finding suggests that the effects of BMP2 on granulosa cell survival may be opposite role to that of BMP4 and BMP7 [202].

1.3.4.6 BMP signaling and cancer

The involvement of TGF-β ligands, including BMPs, on carcinogenesis have not been confirmed as positive or negative. However, several events in cancer development are believed to be associated with BMPs, including metastasis, cancer stem cell development angiogenesis. Similar with the functions of BMPs in embryogenesis [149], BMP2 and BMP4 also promoted EMT in ovarian and breast cancer cells [236, 237] and up-regulated EMT markers, such as *SNAIL* and *SLUG* [236]. Due to the effects of BMPs on stem cell development during embryogenesis, BMP signaling induced cancer stem cell (CSC) differentiation and EMT, resulting in tumorigenesis [238, 239].

1.3.5 Spatiotemporal expression of BMPs in the ovary

According to *in vivo* studies of BMPs in ovary, BMP signaling plays an important role in ovarian folliculogenesis, steroidogenesis and ovulation. These functions may be related to the expression pattern of BMPs in specific cell types in the ovary. A detailed study on spatiotemporal expression patterns of BMPs in rat ovarian cells revealed that BMP2 was mainly expressed in granulosa cells of atretic follicles and lyteolytic corpora lutea. BMP4 was mainly expressed in theca externa cells of dominant follicles and the healthy corpora lutea as well as vascular endothelium. BMP7, similar to BMP4, was expressed in theca interna cells of dominant follicles as well as theca lutein of healthy corpora lutea. BMP6 was expressed in both granulosa cells and oocytes from secondary follicles, granulosa luteal cells in healthy corpora lutea and vascular endothelium. BMP15 was exclusively detected in oocytes from primary follicles [202].

A study of the spatiotemporal expression patterns of BMPs in bovine ovarian cells showed that BMP2 mRNA was mainly expressed in granulosa cells compared to the theca cells with a high level in regressing follicles, which is similar with the expression pattern in rat ovary. BMP4 mRNA was detected in both granulosa and theca cells, while mRNA levels of BMP6 and BMP7 were mainly in theca cells [240]. In the same study, the protein levels of BMP2 and BMP4 appeared more abundant in the granulosa cells, while BMP6 and BMP7 appeared in both granulosa and theca cells [240]. Another study showed that both BMP2 and BMP4 expressed in theca interna layers, as well as oocytes of antral follicles [241].

Similarly, in sheep ovaries, BMP2 mRNA was detected in degenerating granulosa cells of atretic follicles but not in healthy follicles. BMP4 mRNA was detected in surface epithelium and around blood vessels but not in theca or granulosa cells. BMP7 mRNA was detected in rete

tubules but not in granulosa cells, theca cells or oocytes. BMP6 was the only BMP that was detected in oocytes of healthy follicles but not in granulosa or theca cells by RT-qPCR [234].

In pig, the mRNA levels of BMP2 and BMP15 were detected in oocytes, granulosa cells and theca cells, while BMP6 was detected in oocytes and theca cells, but not in granulosa cells. Interestingly, the BMP15 mRNA abundance was negatively correlated with the size of the follicles [242].

The spatiotemporal expression pattern of BMPs in human ovaries is unclear. Studies on BMPs expression pattern in human ovarian cells, by using immunohistochemistry (protein detection) and *in situ* hybridization (mRNA detection), show that BMP4 and BMP7 protein were detected in oocytes, granulosa cells and theca cells , while the mRNAs were detected in fetal oocytes and adult theca cells [243]. Another study showed that the mRNAs of BMP2, BMP4 and BMP6 were detected in corpora lutea including both granulosa-lutein cells and theca-lutein cells [244]. Both mRNA and protein of BMP15 were detected in oocytes, granulosa cells and stoma cells in girls and adults [245]. A study of BMPs expression in human granulosa cells of both healthy and polycystic ovary syndrome (PCOS) women found that the mRNAs of BMP2, BMP4, BMP5, BMP6, BMP7 and BMP8A were detected by RT-qPCR, while the proteins of BMP4, BMP6, and BMP7 were confirmed by immunohistochemistry. BMP6 showed the predominant expression in healthy women and overexpression in PCOS women [246]. Another study suggested that the mRNA levels of BMP2 were detected in granulosa cells of antral follicles but not in the corpus luteum in IVF patients [247].

	Oocytes	Granulosa cells	Theca cells	Other cells	Healthy CL	Lyteolytic CL	Atretic follicles
Rats [202]	BMP6 BMP15	BMP2 BMP6	BMP4 BMP7	BMP3b BMP4 BMP6	BMP3b BMP4	BMP2 BMP4	BMP2 BMP6
Sheep [234] [203]	BMP6 BMP15	BMP6	BMP6	BMP4 BMP7			BMP2
Cow [240] [248] [241]	BMP2 BMP4	BMP2 BMP4 BMP6 BMP7(+/-)	BMP2 BMP4 BMP6 BMP7				
Pig [242]	BMP2 BMP6 BMP15	BMP2 BMP15	BMP2 BMP6 BMP15				
Human [243] [244] [245] [246]	BMP4 BMP7 BMP15	BMP3 BMP4 BMP5 BMP7 BMP15 BMP8A	BMP4 BMP7 BMP15	BMP4	BMP2 BMP4 BMP6	BMP2 BMP4 BMP6	

Table 1-1 Summary of the BMP expression in ovarian cells of different species

Oocytes, granulosa cells and theca cells are from antral follicles; other cells include surface epithelium, stroma cells and vascular endothelium; CL: corpora lutea

1.3.6 The roles of BMP signaling during the periovulatory interval in mouse models

An increasing body of evidence shows that BMPs express in ovarian cells, including oocytes (Table 1), and are involved in regulation of ovulation and luteinization as intrafollicular mediators in animals. Even though, the underlying molecular mechanisms of how BMP signaling regulates the ovulation process is unclear, the knockout mouse models of BMP ligands, receptors and downstream signaling factors (especially SMAD signaling) provide significant implications for understanding the roles of BMPs during the periovulatory interval.

As mentioned previously, *Bmp2*, *Bmp4* and *Bmp7* knockout mice die during embryogenesis [145, 161], or after birth [161]. The mouse ovaries with *Bmp15*$^{-/-}$ are grossly indistinguishable from wild-type ovaries morphologically and histologically. These *Bmp15* null mice are subfertile with reduced ovulation rate (two-thirds the number of oocytes released compared to control) under treatment with a pharmacological superovulation protocol. Moreover, these superovulated mouse ovaries have a percentage of unreleased oocytes (larger than normal but with few cumulus cells surrounding) trapped within luteinized follicles [201]. This phenotype is similar to PGE2 receptor knockouts [249], COX2 knockouts [250] and PR knockouts [95]. Similarly, *Bmp6* null mouse ovaries have no significant differences with wild-type ovaries in follicle numbers and developmental stages. The ovulation rate is significantly decreased with or without injection with hCG *in vivo*. Interestingly, *in vitro* culture of *Bmp6* null COCs, the mRNA levels of hCG-induced EGF-like growth factors and the COC expansion-related genes are significantly decreased, but the EGF-induced COC expansion index is not altered [229]. In summary, *Bmp15* or *Bmp6* knockout mice are subfertile because of fewer oocytes release, but folliculogenesis and the number of preovulatory follicles are like normal.

Based on the fact BMP signaling specificity that is primarily determined by BMP type I receptors (ALK2, ALK3 or ALK6 [251, 252]), ovary-specific BMP receptor knockout mouse studies provide insight into the roles of BMPs in ovarian function. A recent study found that *Alk3* knockout female mice were subfertile with significantly reduced ovulation but normal COC expansion, while *Alk 6* knockout mice exhibited increased multi-oocyte follicles [253]. Another study of mice with deficient *Alk6* showed that the oocytes from *Alk6* mutants can be fertilized *in vitro* but failed to be fertilized *in vivo* because of defects of COC expansion with decreased COX2 expression. These mice also exhibited irregular estrous cycles because of the decreased production of aromatase in granulosa cells [254]. These studies indicated nonredundant functions of ALK3 and ALK6 on ovulation: ALK3 is involved in spontaneous ovulation and ALK6 is essential for COC expansion.

SMAD signaling is the primary signaling pathway induced by TGF-β superfamily members; BMPs activate SMAD1/5/8 as canonical signaling and activate noncanonical SMAD2/3 signaling occasionally in specific cell types. Single knockout mice of *Smad1*, *Smad5* or *Smad8* in ovaries were still fertile [255]. The phenotypes of ovary-specific double knockout mice involving *Smad1* and *Smad5* or triple knockout mice involving *Smad1*, *Smad5* and *Smad8* were infertile and developed metastatic granulosa cell tumors [253, 255]. The study on granulosa cell-specific knockout mice on *Smad2* and *Smad3*, via the *Amhr-2*-cre knock-in allele, indicated that either *Smad2* or *Smad3* silence caused insignificant changes but double null of these two genes resulted in severe infertility with defects of follicular development, ovulation and COC expansion [256]. The study on *Amhr2-Cre* recombinase *Smad4* deletion mice showed increased serum progesterone, decreased number of antral follicles, and premature luteinized follicles with oocytes trapped, as well as severe defects of cumulus-oocytes contacts [257]. Another study on

Cyp19-CreSmad4$^{fl/fl}$ mice showed irregular estrous cycles, deficient ovulation and failure of luteinization [258]. Both studies showed defects of COC expansion in *Smad4* knockouts, which indicates that SMAD signaling is essential for proper ovulation especially in COC expansion.

Chapter 2: Hypothesis and objectives

2.1 Rational

Ovulation is a complex and finely-tuned process and the eventual oocyte rupture from the follicle is only one part of the process. A series of closely-regulated events must occur before oocyte release in preovulatory follicles: COC matrix formation, COC expansion, cell-cell gap junction reduction and oocyte meiosis resumption, and basement membrane breakdown [44] (Figure1-2). Following ovulation, granulosa cells and theca cells left in the follicles undergo luteinization and form the corpus luteum. The ovulatory events must occur before luteinization is complete; otherwise, the oocytes are trapped in luteinized follicles and are destined to degenerate [259]. The appropriate timing and sequential steps of ovulation are controlled by granulosa cells, in which occur a sequence of changes of genes expression related to hormone production, ECM remodeling and intrafollicular mediator production.

Both ovulation and luteinization are induced by LH. The steroid hormones and growth factors secreted by ovarian cells play critical roles in assuring that the ovulation/luteinization process occurs sequentially and properly (Figure1-3). The studies in animal models indicated that failure of any one of the ovulatory events caused by a deficiency of these molecular regulators would prevent ovulation. TGF-β superfamily members, as prime candidates of OSFs, have been demonstrated to be essential to ovulation/luteinization, with correlated roles to do with oocytes: including inhibition of early luteinization and promotion of the COC expansion. The expression of BMPs and BMP receptors has been established in ovarian cells, including oocytes, in many species (Table 1). As intrafollicular molecular regulators, BMP signaling has been demonstrated to be essential to successful ovulation and luteinization in animal models. However,

the detailed functions and underlying mechanisms of BMPs on the ovulation/luteinization process have not been fully established.

2.2 Hypothesis

My overall hypothesis is that during the periovulatory interval, BMP signaling mediates the ovulation/luteinization process by regulating gene expression of steroid hormone production, COC expansion, and follicle ruptures in human granulosa cells.

2.3 Objectives

AIM 1: To investigate the effects of BMPs on steroidogenesis in human granulosa cells.

AIM1A: To examine the effects of BMP4 and BMP7 on progesterone and estradiol production in human granulosa cells.

AIM1B: To examine the effects of BMP4 and BMP7 on StAR, P450scc, 3β-HSD and P450 aromatase expressions in human granulosa cells.

AIM 1C: To investigate which TGF-β type-I receptor is involved in the regulatory effects of StAR by BMP4 and BMP7.

AIM1D: To investigate the downstream signal pathway of BMPs.

AIM 2: To study the contribution of BMPs on COC mass formation in human granulosa cells.

AIM 2A: To examine the effects of BMPs (BMP4, BMP6, BMP7 and BMP15) on HAS1, HAS2, and HAS3 expression.

AIM 2B: To examine which TGF-β receptors are involved in BMP4 up-regulation of HAS2 expression.

AIM 2C: To investigate which SMAD signaling pathway is involved in BMP4 up-regulation of HAS2 expression.

AIM 2D: To investigate the effects of BMPs (BMP4, BMP6, BMP7, and BMP15) on hyaluronan production and hyaluronan binding protein, versican, expression.

AIM 3: To identify the role of BMP4 in ADAMTS-1 expression and activity in human granulosa cells.

AIM 3A: To examine the effect of BMP4 on ADAMTS-1 expression and versican proteolytic cleavage.

AIM 3B: To investigate the role of ADAMTS-1 on versican cleavage.

AIM 3C: To investigate the role of the SMAD pathway being involved in BMP4-regulated ADAMTS-1 activity.

Chapter 3: BMP 4 and 7 suppress basal StAR and progesterone production through ALK3 and SMAD1/5/8/SMAD4 in human granulosa cells

3.1 Introduction

Luteinization, the process whereby granulosa cells and theca cells differentiate into luteal cells, is regulated by LH. The main function of luteal cells is steroid hormone secretion, especially progesterone, to maintain pregnancy or regulate the estrus cycle. Progesterone is derived from cholesterol, which is mainly obtained from blood as low-density lipoprotein and transferred into cells by receptor-mediated endocytosis [24]. StAR governs the process by which cholesterol is transported from the outer to the inner mitochondrial membrane, where cytochrome P450scc catalyzes the first step in progesterone synthesis [25, 33]. Following its conversion from cholesterol by mitochondrial P450scc, pregnenolone is then converted to progesterone by 3βHSD in the smooth endoplasmic reticulum [29]. After ovulation, increasing theca cell vascularization brings more cholesterol to luteinized granulosa cells and high LH levels induce low-density lipoprotein receptor, StAR, P450scc and 3βHSD expression, all of which result in elevated progesterone production to maintain corpus luteum function [21, 30-32]. Throughout this process, growth factors from oocyte, theca and/or granulosa cells exert important paracrine/autocrine regulatory roles on luteinization and progesterone production.

Like other TGF-β superfamily members, such as activins, inhibins, growth differentiation factors and anti-Müllerian hormone, BMPs exert their functions by binding type I and type II receptors which regulate downstream gene expression by phosphorylating SMAD transcription factors [11]. The human genome encodes seven type I receptors known as activin receptor-like kinases (ALK1-7). BMPs are thought to bind ALK2/3/6 leading to the phosphorylation of

SMAD1/5/8, whereas TGF-β superfamily members that bind ALK4/5/7 generally phosphorylate SMAD2/3 [251]. BMPs and their receptors are well-known to be expressed in the reproductive system and to exert important roles in steroidogenesis [202, 260]. BMP4 was found to suppress both basal and FSH-stimulated progesterone production in cow granulosa cells, and to down-regulate FSH-induced StAR and P450scc in sheep granulosa cells [261, 262]. Interestingly, BMP4 and BMP7 suppressed FSH-induced, but not basal, progesterone production in rat granulosa cells [233, 260]. Though accumulating evidence from animal studies suggest that BMP4 and BMP7 can regulate progesterone production, their roles in human granulosa cell steroidogenesis are poorly understood, and the molecular mediators involved have yet to be defined in any species. Therefore in this study, we examined the effects of recombinant BMP4 and BMP7 on progesterone biosynthesis and investigated the underlying mechanisms in human granulosa cells

3.2 Materials and methods

3.2.1 Culture of primary and immortalized human granulosa cells

Primary human granulosa cells were obtained with informed patient consent following approval from the University of British Columbia Research Ethics Board. Follicular aspirates were obtained from 9 women undergoing *in vitro* fertilization, and the controlled ovarian stimulation protocol was as previously described [263]. Granulosa cells were purified by density centrifugation from follicular aspirates as previously described [264]. Briefly, each patient's follicular aspirate was centrifuged at 400 × g for 20min and the cell pellet was resuspended in 4 ml phenol-red free DMEM/F12 medium (Invitrogen, Life Technologies) containing 10% charcoal/dextran-treated fetal bovine serum (Hyclone, GE Healthcare Life Sciences), 1×

Antibiotic-Antimycotic (Gibco, Life Technologies) and 1×Gluta MAX (Gibco, Life Technologies). Cell suspensions were layered on 8 ml Ficoll-Paque Plus (GE Healthcare Life Sciences) and centrifuged at 600 × g for 20min. The granulosa cell layer was removed from the Ficoll-Paque column, washed with medium and resuspended in 5 ml medium. The nontumorigenic immortalized human granulosa cell line (SVOG) was previously produced by transfecting human granulosa cells with the SV40 large T antigen [265]. SVOG cells were passaged using trypsin-EDTA solution (0.05% trypsin, 0.5mM EDTA; Gibco, Life Technologies) when they reached 90% confluence. All cells were counted with a hemocytometer and cell viability was assessed by Trypan blue (0.04%) exclusion. Primary granulosa cells were seeded (1-2 × 10^5 per well) in 12-well plates, while SVOG cells were seeded (2-4 × 10^5 per well) in 6-well plates. Unless otherwise indicated, cells were pre-cultured for 24 hours and then maintained in serum-free medium for 24 hours prior to treatment. Culture medium was changed every other day in all experiments and the cells were cultured in a humidified atmosphere containing 5% CO_2 and 95% air at 37°C.

3.2.2 Antibodies and reagents

Polyclonal rabbit anti-StAR, polyclonal rabbitanti-SMAD1/5/8, and monoclonal mouse anti-α-Tubulin were obtained from Santa Cruz Biotechnology. Polyclonal rabbit anti-phospho-SMAD1/5/8 (Ser$^{463/46}$5, Ser$^{463/465}$, and Ser$^{426/428}$, respectively) and polyclonal rabbit anti-SMAD4 were obtained from Cell Signaling Technology. Horseradish peroxidase-conjugated goat anti-rabbit and goat anti-mouse IgG were obtained from Bio-Rad Laboratories. Recombinant human BMP4, recombinant human BMP7, dorsomorphin dihydrochloride and DMH1 were obtained from R&D Systems. SB-431542 was obtained from Sigma.

3.2.3 Reverse transcription quantitative real-time PCR (RT-qPCR)

Total RNA was extracted with TRIzol Reagent (Invitrogen, Life Technologies) and 2 μg was reverse-transcribed into first-strand cDNA with random primers and MMLV reverse transcriptase (Promega). SYBR Green or TaqMan RT-qPCR was performed on an Applied Biosystems 7300 Real-Time PCR System equipped with 96-well optical reaction plates. Each 20 μl SYBR Green RT-qPCR reaction contained 1× SYBR Green PCR Master Mix (Applied Biosystems), 20 ng cDNA and 250 nM of each specific primer. The primers used were: STAR, 5′-AAACTTACGTGGCTACTCAGCATC-3' (sense) and 5′-GACCTGGTTGATGCTCTTG-3' (antisense); CYP19A1, 5′-GAGAATTCATGCGAGTCTGGA–3' (sense), and 5′-CATTATGTGGAACATACTTGAGGACT–3' (antisense); SMAD4 (SMAD family member 4), 5′-TGGCCCAGGATCACCAGAAG-3' (sense) and 5′-CATCAACACCAATTCCAGCA-3' (antisense); and GAPDH (glyceraldehyde-3-phosphate dehydrogenase), 5′-GAGTCAACGGATTTGGTCGT-3' (sense) and 5′-GACAAGCTTCCCGTTCTCAG-3' (antisense). The specificity of each assay was validated by dissociation curve analysis and agarose gel electrophoresis of PCR products. Alternatively, TaqMan gene expression assays for ACVR (ALK2), BMPR1A (ALK3), BMPR1B (ALK6) and GAPDH (Hs00153836_m1, Hs01034913_g1, Hs00176144_m1 and Hs02758991_g1, respectively; Applied Biosystems) were performed on corresponding cDNA samples. Each 20 μl TaqMan RT-qPCR reaction contained 1 × TaqMan Gene Expression Master Mix (Applied Biosystems), 1 × specific TaqMan gene expression assay, and 20 ng cDNA. Three separate experiments were performed on different cultures and each sample was assayed in triplicate. A mean value was used for the

determination of mRNA levels by the comparative Cq method ($2^{-\Delta\Delta Cq}$) with GAPDH as the reference gene.

3.2.4 Western blot analysis

Cells were seeded, treated as described, and lysed with cell lysis buffer (Cell Signaling Technology) containing protease inhibitor cocktail (Sigma-Aldrich). Extracts were centrifuged at 20,000 x g for 10 min at 4°C to remove cellular debris, and protein concentrations were quantified using the DC Protein Assay (Bio-Rad Laboratories). Equal amounts of protein (50 µg) were separated by 10% SDS-PAGE and transferred to polyvinylidene fluoride membranes. After blocking for 1 hour with 5% nonfat dried milk in tris-buffered saline containing 0.1% Tween 20 (TBST), membranes were incubated overnight at 4°C with primary antibodies diluted 1:1000 in TBST with 5% nonfat dried milk. After washing, membranes were incubated for 1 hour with appropriate peroxidase-conjugated secondary antibody diluted 1:5000 in TBST with 5% nonfat dried milk. Immunoreactive bands were detected using enhanced chemiluminescence reagent or SuperSignal West Femto Chemiluminescence Substrate (Pierce, Thermo Scientific), followed by exposure to CL-XPosure film (Thermo Scientific). Membranes were stripped with stripping buffer (50 mM Tris-HCl pH 7.6, 10 mmol/l β-mercaptoethanol and 1% SDS) at 50°C for 30 min and reprobed with anti-α-Tubulin or anti-SMAD1/5/8 as a loading control.

3.2.5 Small interfering RNA (siRNA) transfection

To knockdown endogenous ALK2, ALK3, ALK6 or SMAD4, cells were seeded (0.5-1 × 10^5 per well in 6-well plates) and transfected the day after seeding with 25 nM ON-TARGET*plus*SMART*pool* siRNAs targeting human ALK2, ALK3, ALK6 or SMAD4 (Dharmacon, GE Healthcare Life Sciences) using Lipofectamine RNAiMAX (Invitrogen, Life Technologies) according to the manufacturer's instructions. ON-TARGET*plus*non-targeting control*pool* siRNA (25 nM) was used as a transfection control in all experiments. Knockdown efficiency was confirmed by RT-qPCR (ALK2, ALK3, ALK6 and SMAD4) and Western blot (SMAD4).

3.2.6 Measurement of progesterone and estradiol

Cells were seeded treated as described, and conditioned medium was stored at -80°C until assayed. Progesterone or estradiol levels in conditioned medium were measured as the manufacturer's instructions using a competitive progesterone or estradiol enzyme immunoassay kit (detection limit, 10 pg/ml and 20 pg/ml, respectively; Cayman Chemical). All samples were measured in triplicate and normalized to total cellular protein content.

3.2.7 Statistical analysis

Results are presented as the mean ± SEM of at least three independent experiments performed on separate cultures. PRISM software (GraphPad Software Inc.) was used to perform one-way ANOVA followed by Tukey's test for multiple comparisons of means. Means were considered statistically different if $P < 0.05$ and are indicated by different letters.

3.3 Results

3.3.1 BMP4 and BMP7 suppress StAR expression in human granulosa cells

To examine the effects of BMP4 and BMP7 on granulosa cell steroidogenesis, we first used RT-qPCR and Western blot to examine their effects on the expression of StAR, which regulates the rate-limiting step in steroid biosynthesis. StAR mRNA and protein levels were measured in SVOG immortalized human granulosa cells following treatment with vehicle control or 30 ng/ml recombinant human BMP4 or BMP7 for 3, 6, 12 or 24 hours. The RT-qPCR results show that treatment with either BMP4 (Figure 3-1A) or BMP7 (Figure 3-1B) down-regulated StAR mRNA levels after 12 and 24 hours. The suppressive effects of BMP4 and BMP7 on StAR protein levels were further confirmed by Western blot analysis (Figure 3-1C and 3-1D). To evaluate the concentration-dependent effects of BMP4 and BMP7 on StAR expression, SVOG cells were treated for 24 hours with varying concentrations of each protein (1, 10, 30 or 100 ng/ml) and StAR mRNA and protein levels were measured by RT-qPCR and Western blot, respectively. Treatment with either BMP4 or BMP7 down-regulated StAR mRNA (Figure 3-2A and 3-2B) and protein (Figure 3-2C and 3-2D) levels in a concentration-dependent manner. Interestingly, treatment with 1 ng/ml BMP4 induced significant decreases in StAR expression whereas only concentrations of 30 or 100 ng/ml of BMP7 yielded significant effects at both the mRNA and protein level.

To further confirm the suppressive effects of BMP4 and BMP7 on StAR expression, primary human granulosa cells were treated for 24 hours with 30 or 50 ng/ml BMP4 or BMP7. As shown in Figure 3-2E, treatment with 30 ng/ml BMP4 significantly reduced StAR mRNA levels, while treatment with BMP7 yielded reductions approaching statistical significance.

However, when the treatment concentrations were increased to 50 ng/ml, both BMP4 and BMP7 significantly reduced StAR mRNA levels (Figure 3-2F).

3.3.2 BMP4 and BMP7 down-regulate StAR via ALK3 in SVOG cells

Though both type I and type II receptors are required for BMP signaling, signaling specificity is primarily determined by BMP type I receptors (ALK2, ALK3 and/or ALK6; [251, 252]). To determine which ALK(s) is required for the suppression of StAR expression by BMP4 and BMP7 in human granulosa cells, SVOG cells were treated for 24 hours with BMP4 or BMP7 (30 ng/ml) in the presence or absence of dorsomorphin (10 μM), DMH1 (0.25 μM) or SB-431542 (10 μM). Dorsomorphin is a selective inhibitor of ALK2/3/6 [266], DMH1 is a specific inhibitor of ALK2/3 [267], and SB-431542 is a selective inhibitor ALK4/5/7 (activin/TGF-β type I receptors; [268]). The suppressive effects of BMP4 and BMP7 on StAR mRNA and protein levels were abolished by pre-treatment with either dorsomorphin (Figure 3-3A and 3-3D) or DMH1 (Figure 3-3B and 3-3E), but not SB-431542 (Figure 3-3C and 3-3F). These results suggest that BMP type I receptors ALK2 or ALK3, but not ALK6, are involved in the inhibitory effects of BMP4 and BMP7 on StAR expression in human granulosa cells.

Next, we used an siRNA-based approach to confirm these findings and further specify which ALK(s) mediates the effects of BMP4 and BMP7 on StAR expression. SVOG cells were pre-treated for 48 hours with siRNA targeting ALK2, ALK3 or ALK6, treated for a further 24 hours with 30 ng/ml BMP4 or BMP7, and StAR mRNA and protein levels were measured by RT-qPCR and Western blot. As shown in Figure 3-4A-C, transfection with each siRNA significantly reduced the mRNA levels of only the targeted ALK. Interestingly, the suppressive effects of BMP4 and BMP7 on StAR mRNA and protein levels were abolished by knockdown of

ALK3, but not ALK2 or ALK6 (Figure 3-4D-I). Taken together, these results indicate that ALK3 is required for the down-regulation of StAR expression by BMP4 and BMP7 in SVOG cells.

3.3.3 SMAD1/5/8-SMAD4 signaling is required for BMP4- and BMP7-induced down-regulation of StAR in SVOG cells

We next investigated whether canonical SMAD1/5/8-SMAD4 signaling mediates the effects of BMP4 and BMP7 on StAR expression. First, we used Western blot to measure phosphorylated and total SMAD1/5/8 levels in SVOG cells treated for 60 minutes with BMP4 or BMP7 (30 ng/ml) in the presence or absence of DMH1 (0.25 μM). As shown in Figure 3-5A, pre-treatment with DMH1 inhibited BMP4- and BMP7-induced SMAD1/5/8 phosphorylation. Next, we examined BMP4- and BMP7-induced SMAD1/5/8 phosphorylation in SVOG cells pre-treated for 48 hours with or without ALK3 siRNA. As shown in Figure 3-5B, knockdown of ALK3 reversed the phosphorylation of SMAD1/5/8 by BMP4 and BMP7.

Upon phosphorylation/activation, SMAD1/5/8 binds with common SMAD4 to form heteromeric complexes that regulate target gene transcription. To determine whether the effects of BMP4 and BMP7 on StAR expression are SMAD-dependent, siRNA was used to down-regulate SMAD4 prior to treatment for 24 hours with BMP4 or BMP7 (30 ng/ml). As shown in Figure 3-5C and 3-5D, pre-treatment for 48 hours with SMAD4 siRNA significantly reduced SMAD4 mRNA and protein levels, and abolished BMP4- and BMP7-induced down-regulation of StAR mRNA and protein. Taken together, these results indicate that SMAD1/5/8-SMAD4 signaling is involved in the suppression of StAR expression by BMP4 and BMP7 in SVOG cells.

3.3.4 BMP4 and BMP7 decrease progesterone production in human granulosa cells

The effects of BMP4 and BMP7 on granulosa cell progesterone production were examined in SVOG cells treated for 24 hours with vehicle control or increasing concentrations (1, 10, 30 or 100 ng/ml) of BMP4 or BMP7. Treatment with either BMP4 (Figure 3-6A) or BMP7 (Figure 3-6B) down-regulated progesterone accumulation in conditioned medium in a concentration-dependent manner. Consistent with the findings for StAR expression, BMP4 induced significant decreases at all concentrations tested whereas only treatment with 30 or 100 ng/ml BMP7 yielded significant effects on progesterone production. Next, we investigated the effects of BMP4 and BMP7 on progesterone production in primary human granulosa cells. As shown in Figure 3-6C and 3-6D, progesterone accumulation in conditioned medium was significantly decreased by treatment with 30 or 50 ng/ml BMP4, and 50 ng/ml BMP7.

3.3.5 BMP4 and BMP7 increase aromatase mRNA levels and estradiol production in primary human granulosa cells

Next, we investigated the effects of BMP4 and BMP7 on aromatase mRNA and estradiol production in primary human granulosa cells. As shown in Figure 3-7, treatment for 24 hours with 30 ng/ml BMP4 or BMP7 significantly increased aromatase mRNA levels and estradiol accumulation in conditioned medium.

3.4 Discussion

We describe for the first time the effects of recombinant human BMP4 or BMP7 on StAR expression and progesterone production in primary and immortalized human granulosa cells. Specifically, treatment with BMP4 or BMP7 down-regulated the rate-limiting regulatory protein StAR and suppressed progesterone accumulation in conditioned medium. Following ovulation, granulosa cells become part of the corpus luteum which produces large amounts of progesterone that are required for establishing and maintaining early pregnancy. However, elevated progesterone levels prior to ovulation can result in premature luteinization which adversely affects oocyte quality and endometrial receptivity [269, 270]. Though pituitary gonadotropins are key regulators of luteal steroidogenesis [271-273], important paracrine or autocrine regulation has also been described for local growth factors, such as IGF, TGF-β and activins/inhibins [6, 274]. In addition to the well-characterized roles of oocyte-derived factors (e.g. BMP15 and growth differentiation factor 9 [118, 275]), increasing evidence suggests that other intrafollicular BMPs also play important roles in follicular development and granulosa cell function.

The present results suggest BMP4 and BMP7 act as inhibitors of granulosa cell luteinization prior to or after ovulation. Indeed, injection of BMP7 into the ovarian bursa of rats significantly reduces serum progesterone levels [233]. Studies in cow and sheep granulosa cells have demonstrated suppressive effects of BMP4 and BMP7 on basal progesterone production [234, 248, 261, 276, 277], whereas in rat granulosa cells both no effects [260] and suppression [234] have been reported. In addition to regulating basal progesterone synthesis, BMP4 and BMP7 have been shown to suppress the production of progesterone in response to FSH in rat granulosa cells [260], IGF-I in cow granulosa cells [248], and cAMP in KGN human granulosa tumor cells [40]. Moreover, treatment with BMP4 suppresses FSH-induced progesterone

production in sheep granulosa cells [261]. Future studies will be required to investigate the effects of BMP4 and BMP7 on gonadotropin-induced progesterone production in human granulosa cells. Little is known about the functional roles of BMPs in the luteal phase, especially as it relates to corpus luteum formation, function and regression. Interestingly, treatment of cow theca cells with BMP4 and BMP7 enhances basal, but not LH-induced, progesterone production [278]. Whether the differential effects of BMP4 and BMP7 on progesterone production are maintained in granulosa- vs. theca-luteal cells of the corpus luteum would be an interesting question for future investigation.

BMP4 and BMP7 inhibit progesterone production in human granulosa cells by down-regulating the mRNA and protein levels of StAR. In rat granulosa cells, treatment with BMP7 suppressed FSH-induced StAR mRNA levels, but did not alter basal mRNA levels [233]. BMP4 has been shown to suppress basal StAR mRNA levels in cow granulosa cells [276], as well as basal and FSH-induced StAR mRNA and protein levels in sheep granulosa cells [261]. In KGN cells, BMP4 and BMP7 were shown to reduce basal and cAMP-induced StAR mRNA levels as well as basal *STAR* promoter activity [40]. Though BMP4 and BMP7 can regulate StAR gene expression, the mechanisms underlying these effects remain poorly defined. We have demonstrated that the effects of BMP4 and BMP7 on StAR expression are SMAD-dependent; however, the time-course (≥12 hours) suggests indirect effects via induction/suppression of other transcription factors. This is in keeping with the fact that no consensus SMAD binding elements have been described for the *STAR* promoter. However, a multitude of associations with diverse DNA-binding factors is critical to selective and high affinity interactions of SMAD complexes with target gene promoters [279, 280]. Previous studies in sheep granulosa cells demonstrated that the induction of *STAR* promoter activity by SF-1 overexpression was inhibited by co-

transfection with SMAD1 [261]. Though it suggests a negative crosstalk between SMAD1 and SF-1, co-immunoprecipitation studies failed to detect any direct binding between SMAD1 and SF-1 [261]. In addition, down-regulation of SF-1 expression is not likely to contribute to the effects of BMP4 or BMP7 on StAR expression as neither BMP altered SF-1 mRNA levels in SVOG cells (Figure 3-8). Regulatory SMADs have also been shown to interact with transcription factors such as activator protein 1 and CCAAT enhancer binding protein β [280], both of which have also been implicated in the regulation of *STAR* promoter activity [281]. In addition, BMP4 has been shown to reduce the levels of acetylated histone H3 in the *STAR* promoter at 48 and 72 hours in cow granulosa cells [276]. Clarifying the mechanism(s) by which SMAD complexes regulate StAR expression in human granulosa cells is of increasing interest given that, in addition to the present findings for BMP4 and BMP7, studies have implicated BMP2 [247], BMP15 [118], activins [282] and TGF-β1 [117] in the regulation of StAR.

In addition to characterizing the effects of BMP4 and BMP7 on StAR expression and progesterone production, we used pharmacological and siRNA-based approaches to demonstrate that ALK3 is required for the down-regulation of StAR expression in human granulosa cells. These results are consistent with our previous studies showing that ALK3 mediates the effects of BMP15 on StAR expression in human granulosa cells [118]. However, they differ from our recent findings that differential subsets of BMP type I receptors mediate the down-regulation of pentraxin 3 by BMP4 (ALK3/6) and BMP7 (ALK3/2) in human granulosa cells [283]. Together, these findings suggest that human granulosa cells have the capacity to signal via multiple type I receptors in response to BMP4 or BMP7, but that different receptor(s) may somehow be coupled to different target genes and/or functions. Previous studies in diverse cell types have described differing type I receptor binding affinities for BMP4 and BMP7 [137, 284]. Our results appear to

support differences in receptor binding characteristics as BMP4 produced significant reductions in StAR expression and progesterone production at 1 ng/ml, whereas concentrations between 30-100 ng/ml were required for BMP7. Nevertheless, previous studies in KGN cells have likewise demonstrated differences in type I receptor dependence between multiple promoter-luciferase constructs [40]. In particular, forced-expression of ALK3 or ALK6 enhanced the effects of BMP4, BMP7 or BMP6 on the 3TP-luciferase construct. Overexpression of ALK3 or ALK6 also enhanced the effects of BMP7 or BMP6 on the Id1- and Tlx2-luciferase constructs; whereas the effects of BMP4 on the Tlx2-luciferase construct were only enhanced by ALK3, and its effects on the Id1-luciferase construct were not enhanced by overexpression of ALK2, ALK3 or ALK6 [40]. Future studies investigating putative differential coupling of BMP type I receptors to specific cellular responses, as well as the mechanisms involved, are warranted as they could lead to novel pharmacological strategies for clinical use. Though selective antagonists or agonists of individual BMP type I receptors have yet to be fully developed, they might be used to selectively target specific biological processes. For example in granulosa cells, specific targeting of ALK6 could alter the effects of BMP4, but not BMP7, on pentraxin 3 (and possibly cumulus expansion) without altering the effects of either protein on StAR expression or progesterone production.

In summary, the present study demonstrates that BMP4 and BMP7 suppress progesterone production by down-regulating the expression of StAR, but increase estradiol production by up-regulating the mRNA of aromatase in human granulosa cells. Furthermore, our results suggest that the suppressive effects of BMP4 and BMP7 on StAR are mediated by ALK3 with canonical SMAD1/5/8 signaling involved. These findings provide important insights into the functional roles and molecular mechanisms of BMPs in the periovulatory period.

3.5 Figures

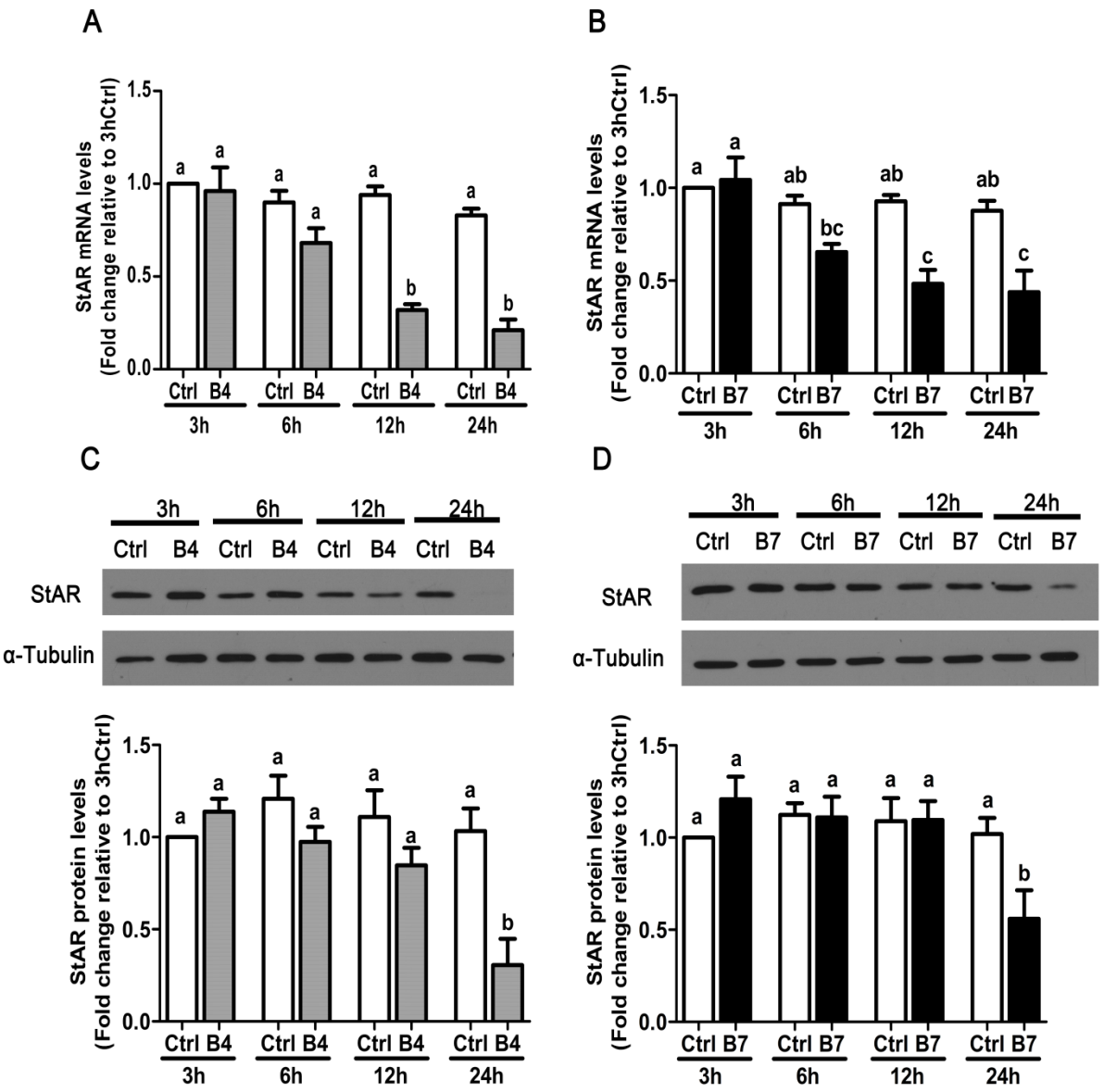

A
StAR mRNA levels
(Fold change relative to 3hCtrl)
1.5
1.0
0.5
0.0
a a a a a b a b
Ctrl B4 Ctrl B4 Ctrl B4 Ctrl B4
3h 6h 12h 24h
B
StAR mRNA levels
(Fold change relative to 3hCtrl)
a a ab bc ab c ab c
Ctrl B7 Ctrl B7 Ctrl B7 Ctrl B7
3h 6h 12h 24h
C
3h 6h 12h 24h
Ctrl B4 Ctrl B4 Ctrl B4 Ctrl B4
StAR
α-Tubulin
StAR protein levels
(Fold change relative to 3hCtrl)
a a a a a a a b
Ctrl B4 Ctrl B4 Ctrl B4 Ctrl B4
3h 6h 12h 24h
D
3h 6h 12h 24h
Ctrl B7 Ctrl B7 Ctrl B7 Ctrl B7
StAR
α-Tubulin
StAR protein levels
(Fold change relative to 3hCtrl)
a a a a a a a b
Ctrl B7 Ctrl B7 Ctrl B7 Ctrl B7
3h 6h 12h 24h

Figure 3-1 Time-dependent effects of BMP4 and BMP7 on StAR expression in SVOG immortalized human granulosa cells.

Cells were treated with vehicle control (Ctrl) or 30 ng/ml BMP4 (B4; A and C) or BMP7 (B7; B and D) for 3, 6, 12or 24 hours, and StAR mRNA (A and B) and protein (C and D) levels were examined by RT-qPCR and Western blot, respectively. GAPDH mRNA levels and α-Tubulin protein levels were used as internal controls. Results are expressed as the mean ± SEM of at least three independent experiments and values marked by different letters are significantly different (P<0.05).

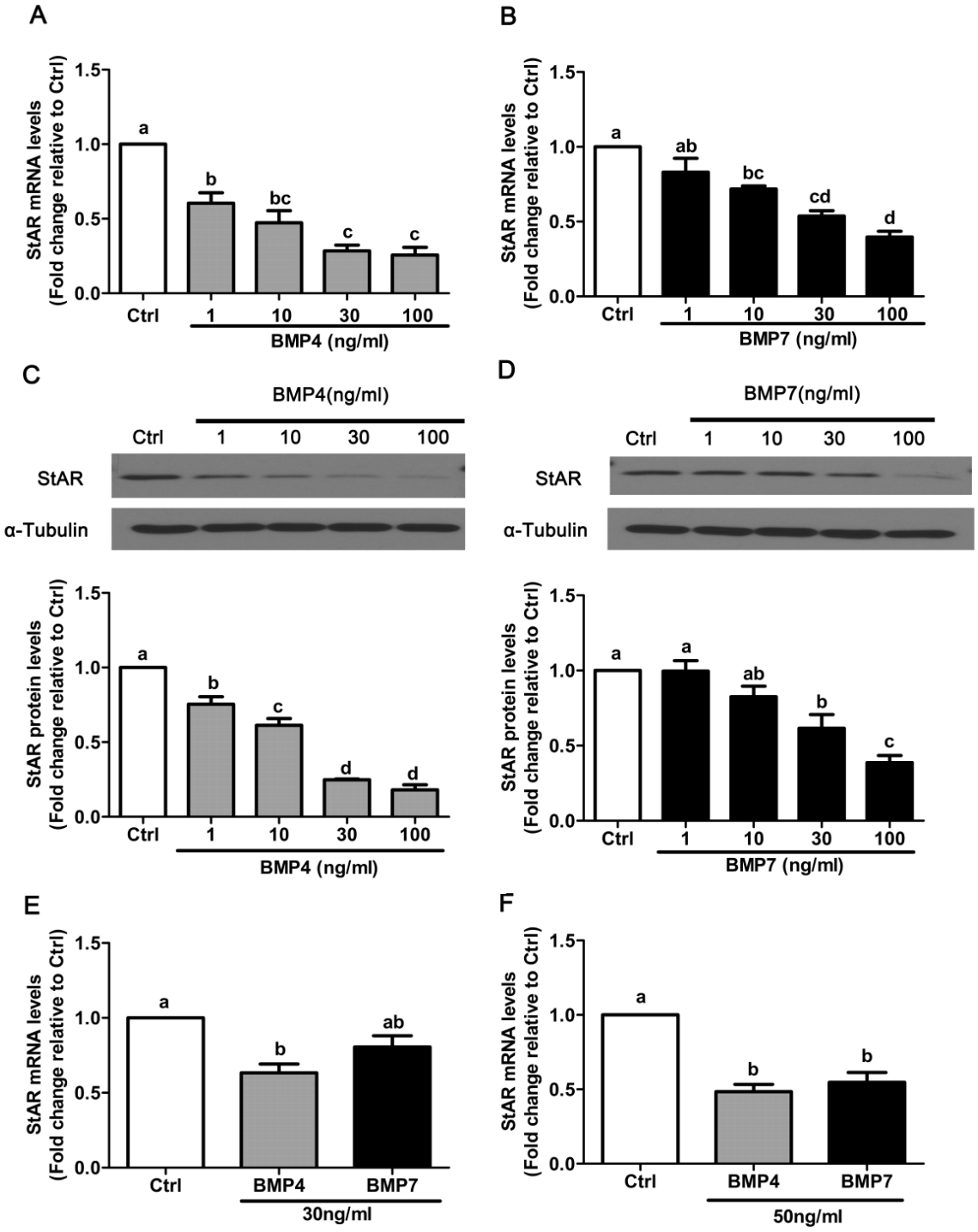

A
StAR mRNA levels (Fold change relative to Ctrl)
1.5
1.0
0.5
0.0
a
b
bc
c
c
Ctrl
1
10
30
100
BMP4 (ng/ml)
B
StAR mRNA levels (Fold change relative to Ctrl)
a
ab
bc
cd
d
Ctrl
1
10
30
100
BMP7 (ng/ml)
C
BMP4(ng/ml)
Ctrl
1
10
30
100
StAR
α-Tubulin
StAR protein levels (Fold change relative to Ctrl)
a
b
c
d
d
Ctrl
1
10
30
100
BMP4 (ng/ml)
D
BMP7(ng/ml)
Ctrl
1
10
30
100
StAR
α-Tubulin
StAR protein levels (Fold change relative to Ctrl)
a
a
ab
b
c
Ctrl
1
10
30
100
BMP7 (ng/ml)
E
StAR mRNA levels (Fold change relative to Ctrl)
a
b
ab
Ctrl
BMP4
BMP7
30ng/ml
F
StAR mRNA levels (Fold change relative to Ctrl)
a
b
b
Ctrl
BMP4
BMP7
50ng/ml

Figure 3-2 BMP4 and BMP7 down-regulate StAR expression in a concentration-dependent manner in primary and SVOG immortalized human granulosa cells.

A-D, SVOG immortalized human granulosa cells were treated for 24 hours with vehicle control (Ctrl) or increasing concentrations (1, 10, 30 or 100 ng/ml) of BMP4 (A and C) or BMP7 (B and D) and StAR mRNA (A and B) and protein (C and D) levels were examined by RT-qPCR and Western blot, respectively. E and F, Primary human granulosa cells were treated for 24 hours with 30 ng/ml (E) or 50 ng/ml (F) BMP4 or BMP7 and StAR mRNA levels were examined by RT-qPCR. GAPDH mRNA levels and α-Tubulin protein levels were used as internal controls. Results are expressed as the mean ± SEM of at least three independent experiments and values marked by different letters are significantly different ($P<0.05$).

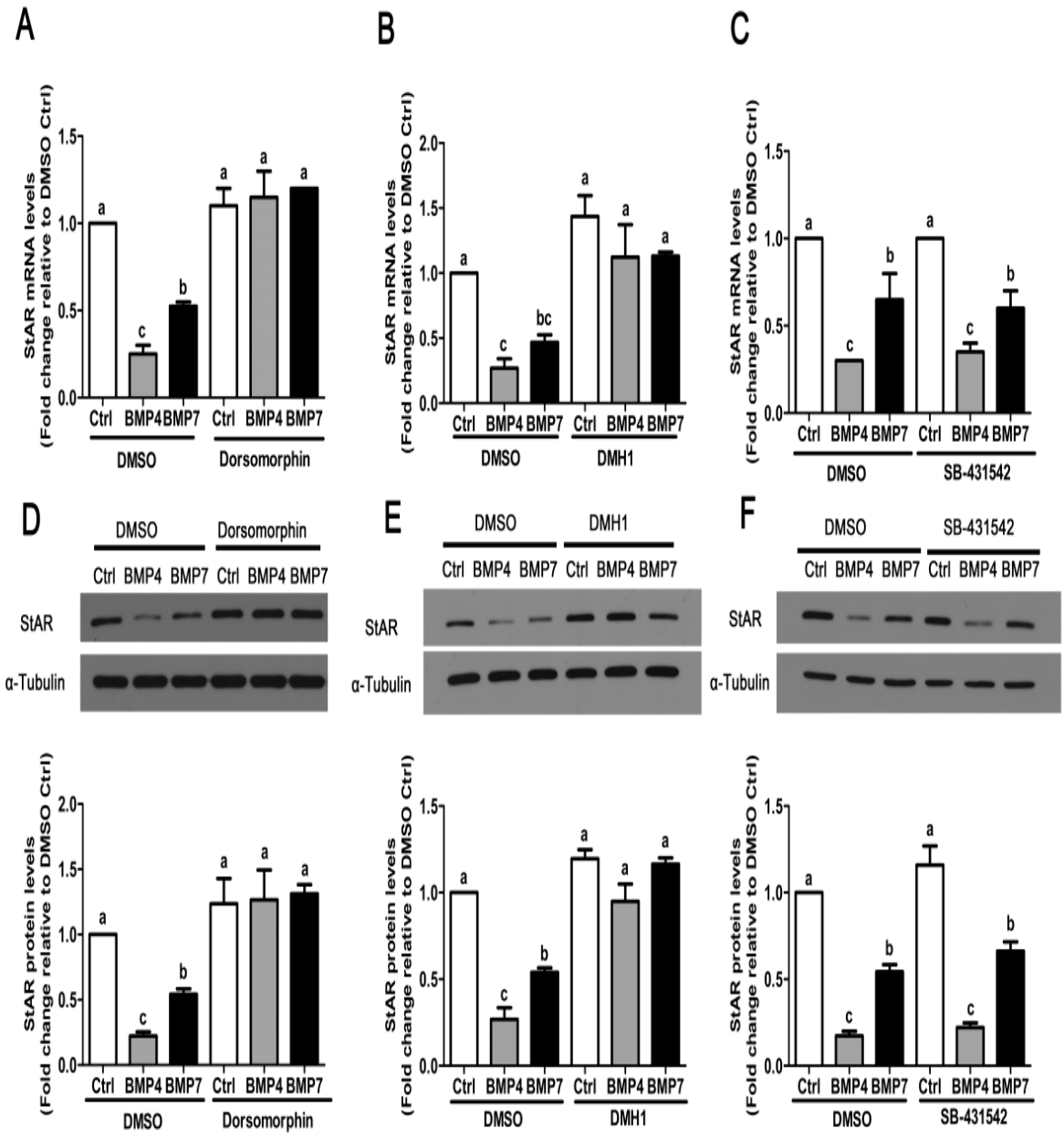

A
B
C
StAR mRNA levels
(Fold change relative to DMSO Ctrl)
Ctrl BMP4 BMP7 Ctrl BMP4 BMP7
DMSO
Dorsomorphin
DMH1
SB-431542
D
E
F
StAR
α-Tubulin
StAR protein levels
(Fold change relative to DMSO Ctrl)

Figure 3-3 The effects of BMP4 and BMP7 on StAR expression are abolished by BMP type I receptor inhibitors dorsomorphin and DMH1, but not by TGF-β type I receptor inhibitor SB-431542 in SVOG cells.

SVOG immortalized human granulosa cells were treated for 24 hours with vehicle control (Ctrl) or 30 ng/ml BMP4 or BMP7 in the absence (DMSO) or presence of 10 μM dorsomorphin (A and D), 0.25 μM DMH1 (B and E) or 10 μM SB- 431542 (C and F). StAR mRNA (A-C) and protein (D-F) levels were examined by RT-qPCR and Western blot, respectively. GAPDH mRNA levels and α-Tubulin protein levels were used as internal controls. Results are expressed as the mean ± SEM of at least three independent experiments and values marked by different letters are significantly different ($P<0.05$).

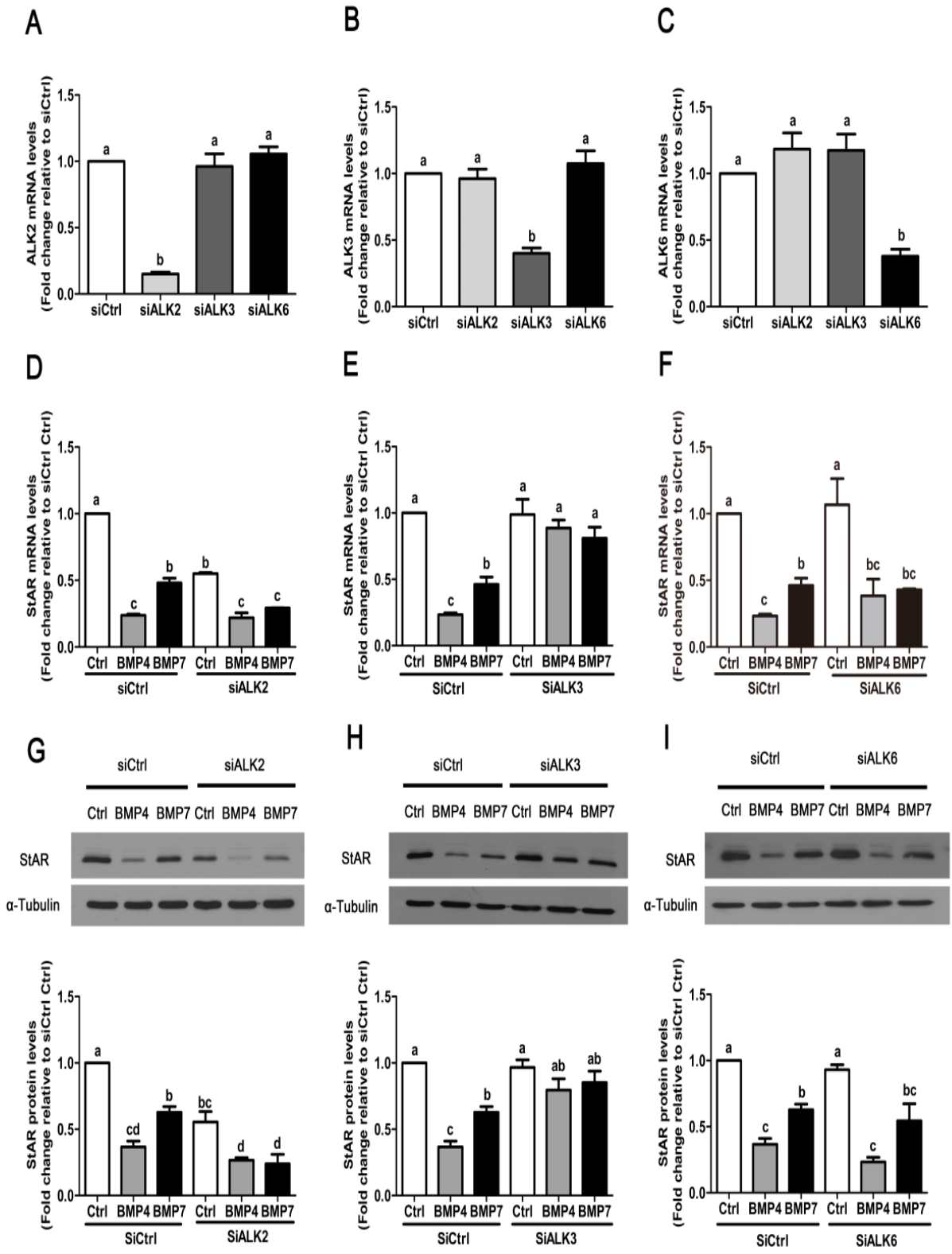
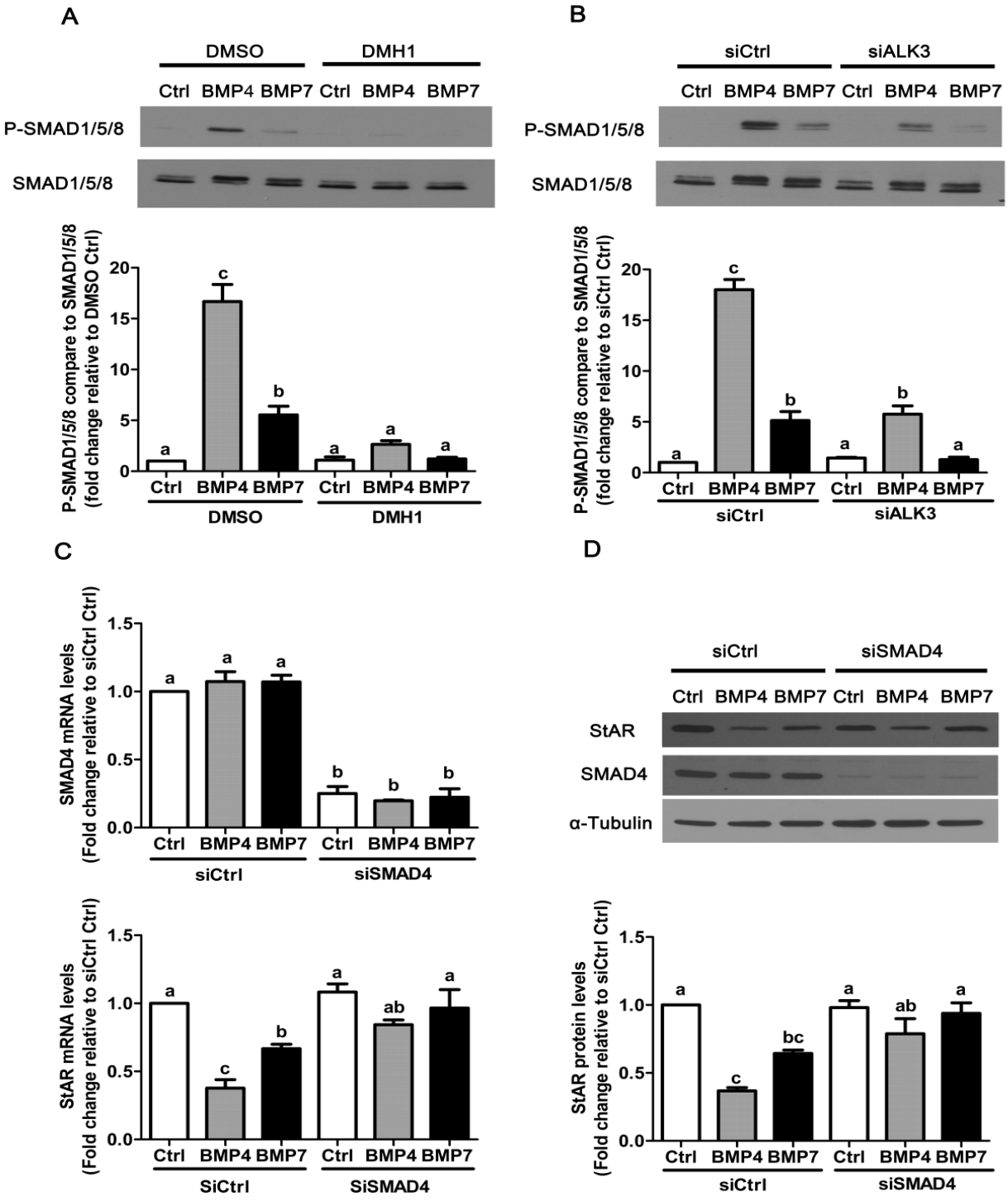

A
ALK2 mRNA levels (Fold change relative to siCtrl)
siCtrl siALK2 siALK3 siALK6
B
ALK3 mRNA levels (Fold change relative to siCtrl)
siCtrl siALK2 siALK3 siALK6
C
ALK6 mRNA levels (Fold change relative to siCtrl)
siCtrl siALK2 siALK3 siALK6
D
StAR mRNA levels (Fold change relative to siCtrl Ctrl)
Ctrl BMP4 BMP7 Ctrl BMP4 BMP7
siCtrl siALK2
E
StAR mRNA levels (Fold change relative to siCtrl Ctrl)
Ctrl BMP4 BMP7 Ctrl BMP4 BMP7
SiCtrl SiALK3
F
StAR mRNA levels (Fold change relative to siCtrl Ctrl)
Ctrl BMP4 BMP7 Ctrl BMP4 BMP7
SiCtrl SiALK6
G
siCtrl siALK2
Ctrl BMP4 BMP7 Ctrl BMP4 BMP7
StAR
α-Tubulin
StAR protein levels (Fold change relative to siCtrl Ctrl)
Ctrl BMP4 BMP7 Ctrl BMP4 BMP7
SiCtrl SiALK2
H
siCtrl siALK3
Ctrl BMP4 BMP7 Ctrl BMP4 BMP7
StAR
α-Tubulin
StAR protein levels (Fold change relative to siCtrl Ctrl)
Ctrl BMP4 BMP7 Ctrl BMP4 BMP7
SiCtrl SiALK3
I
siCtrl siALK6
Ctrl BMP4 BMP7 Ctrl BMP4 BMP7
StAR
α-Tubulin
StAR protein levels (Fold change relative to siCtrl Ctrl)
Ctrl BMP4 BMP7 Ctrl BMP4 BMP7
SiCtrl SiALK6

Figure 3-4 BMP4 and BMP7 down-regulate StAR via ALK3 in SVOG immortalized human granulosa cells.

Cells were transfected for 48 hours with 25nM control siRNA (siCtrl), ALK2 siRNA (siALK2), ALK3 siRNA (siALK3) or ALK6 siRNA (siALK6), and then treated for a further 24 hours with vehicle control (Ctrl) or 30 ng/ml BMP4 or BMP7. The efficiency and specificity of knockdown for each siRNA was evaluated by RT-qPCR (A-C). The mRNA (D-F) and protein (G-I) levels of StAR were examined by RT-qPCR and Western blot, respectively. GAPDH mRNA levels and α-Tubulin protein levels were used as internal controls. Results are expressed as the mean ± SEM of at least three independent experiments and values marked by different letters are significantly different (P<0.05).


A
DMSO
DMH1
Ctrl BMP4 BMP7 Ctrl BMP4 BMP7
P-SMAD1/5/8
SMAD1/5/8
P-SMAD1/5/8 compare to SMAD1/5/8 (fold change relative to DMSO Ctrl)
Ctrl BMP4 BMP7 Ctrl BMP4 BMP7
DMSO
DMH1
B
siCtrl
siALK3
Ctrl BMP4 BMP7 Ctrl BMP4 BMP7
P-SMAD1/5/8
SMAD1/5/8
P-SMAD1/5/8 compare to SMAD1/5/8 (fold change relative to siCtrl Ctrl)
Ctrl BMP4 BMP7 Ctrl BMP4 BMP7
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SMAD4 mRNA levels (Fold change relative to siCtrl Ctrl)
Ctrl BMP4 BMP7 Ctrl BMP4 BMP7
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StAR mRNA levels (Fold change relative to siCtrl Ctrl)
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SiCtrl
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Ctrl BMP4 BMP7 Ctrl BMP4 BMP7
StAR
SMAD4
α-Tubulin
StAR protein levels (Fold change relative to siCtrl Ctrl)
Ctrl BMP4 BMP7 Ctrl BMP4 BMP7
siCtrl
siSMAD4

Figure 3-5 SMAD1/5/8-SMAD4 signaling is required for BMP4- and BMP7-induced down-regulation of StAR in SVOG immortalized human granulosa cells.

A, Phosphorylated SMAD1/5/8 protein levels were examined by Western blot following treatment of cells for 60 min with vehicle control (Ctrl) or 30 ng/ml BMP4 or BMP7 in the absence (DMSO) or presence of DMH1 (0.25 μM). B, Cells were transfected for 48 hours with 25 nM control siRNA (siCtrl) or ALK3 siRNA (siALK3), treated for 60 min with 30 ng/ml BMP4 or BMP7, and phosphorylated SMAD1/5/8 protein levels were examined by Western blot. C and D, Cells were transfected for 48 hours with 25nM control siRNA (siCtrl) or SMAD4 siRNA (siSMAD4) and then treated for a further 24 hours with BMP4 or BMP7 (30 ng/ml). SMAD4 and StAR mRNA (C) and protein (D) levels were examined by RT-qPCR and Western blot, respectively. GAPDH mRNA levels and α-Tubulin protein levels were used as internal controls. Results are expressed as the mean ± SEM of at least three independent experiments and values marked by different letters are significantly different (P<0.05).

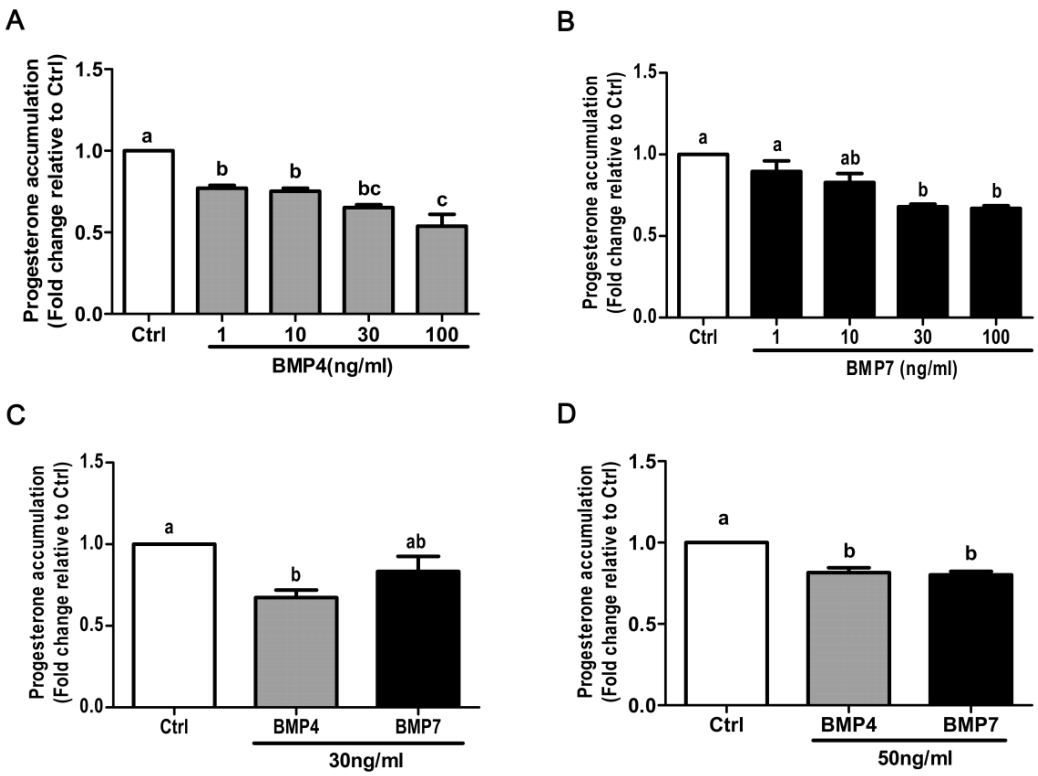

Figure 3-6 BMP4 and BMP7 suppress progesterone accumulation in primary and SVOG immortalized human granulosa cells.

A and B, SVOG immortalized human granulosa cells were treated for 24 hours with vehicle control (Ctrl) or increasing concentrations (1, 10, 30 or 100 ng/ml) of BMP4 (A) or BMP7 (B) and progesterone accumulation in conditioned medium was measured by ELISA. C, Primary human granulosa cells were treated with 30 (C) or 50 ng/ml (D) BMP4 or BMP7 for 24 hours and progesterone accumulation was measured by ELISA. Results are expressed as the mean ± SEM of at least three independent experiments and values marked by different letters are significantly different (P<0.05).

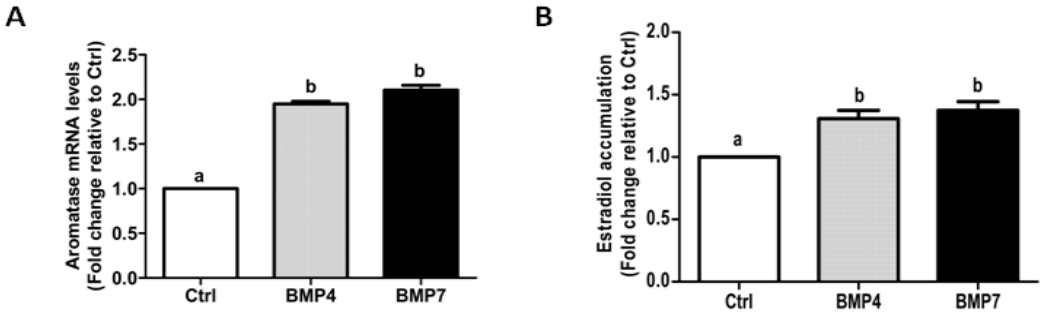

Figure 3-7 BMP4 and BMP7 increase aromatase expression and estradiol accumulation in primary human granulosa cells.

A and B, Primary human granulosa cells were treated with 30 ng/ml BMP4 or BMP7 for 24 hours and aromatase mRNA levels (A) and estradiol accumulation (B) were measured by RT-qPCR and ELISA, respectively. GAPDH mRNA levels were used as an internal control. Results are expressed as the mean ± SEM of at least three independent experiments and values marked by different letters are significantly different ($P<0.05$).

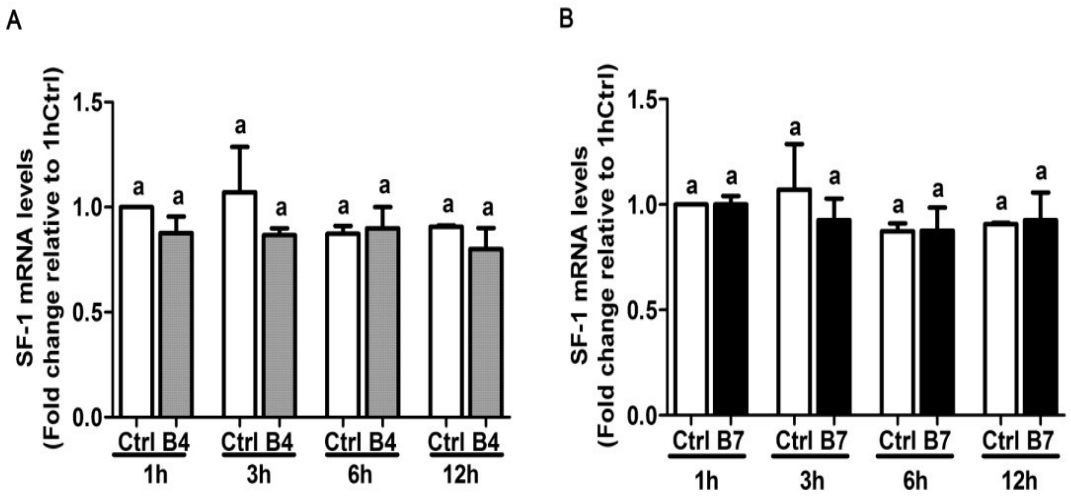

Figure 3-8 Neither BMP4 nor BMP7 affects the mRNA levels of SF-1 in SVOG immortalized human granulosa cells.

Cells were treated with vehicle control (Ctrl) or 30 ng/ml BMP4 (B4; A) or BMP7 (B7; B) for 1, 3, 6 or 12 hours, and SF-1 mRNA levels were examined by RT-qPCR. GAPDH mRNA levels were used as an internal. Results are expressed as the mean ± SEM of at least three independent experiments and values without common letters are significantly different ($P<0.05$).

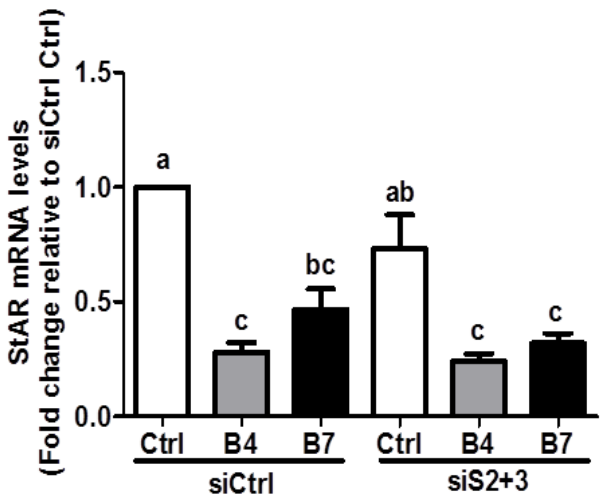

Figure 3-9 Neither BMP4- nor BMP7-induced down-regulation of StAR is affected by knockdown of both SMAD2 and SMAD3 in SVOG immortalized human granulosa cells.

Cells were transfected with 25 nM control siRNA (siCtrl) or both SMAD2 and SMAD3 siRNA (siS2+3) for 72 hours, and then treated with BMP4 or BMP7 (30 ng/ml) for 24 hours. The mRNA levels of StAR were examined by RT-PCR. GAPDH mRNA levels were used as an internal control. Results are expressed as the mean ± SEM of at least three independent experiments and values marked by different letters are significantly different ($p<0.05$).

Chapter 4: Differential activation of noncanonical SMAD2/SMAD3 signaling by BMPs causes disproportionate induction of hyaluronan production in human granulosa cells

4.1 Introduction

After the LH surge, the oocyte and its surrounding cumulus cells assemble a unique matrix called the cumulus-oocyte complex (COC). Appropriate COC matrix expansion is obligatory for ovulation, oocyte passage through the oviduct and fertilization [285, 286]. Hyaluronan, a ubiquitous ECM component, composes the backbone of the COC matrix in vertebrates and is essential for successful ovulation [46]. Three types of hyaluronan synthases (HASs) are expressed in mammalian cells to catalyze the hyaluronan biosynthetic reaction: HAS1, HAS2, and HAS3 [47]. *Has2* gene target deletion embryos die because of severe cardiac-vascular deficiency, whereas *Has1* or *Has3* knockout mice are viable and fertile [287-290]. Compared to HAS1 and 3, HAS2 synthesizes an extremely large hyaluronan and plays a critical role in hyaluronan synthesis during COC expansion [48]. Animal studies on different tissues have revealed that synthesis of hyaluronan and activity of HAS2 are stimulated by growth factor signaling [112, 291, 292]. The role of hyaluronan is to fill the intercellular spaces of the COC matrix, which is stabilized and regulated by other COC expansion-related proteins: TSG-6 [293], pentraxin 3 (PTX3) [294] and prostaglandin synthase 2 (PTGS2) [109, 295] and proteoglycans [296].

Bone morphogenetic proteins (BMPs) belong to the transforming growth factor-β (TGF-β) superfamily, which also includes TGF-β, activin, inhibins, growth differentiation factors (GDFs) and anti-Müllerian hormone. TGF-β family members exert their functions by binding to

type I activin receptor-like kinase (ALK) and type II receptors and regulate downstream gene expression by phosphorylating SMAD signaling [11]. BMPs and anti-Müllerian hormone activate SMAD 1/5/8/ though ALK2, ALK3, and ALK6, whereas TGF-β, GDFs, Activins and nodal phosphorylate SMAD2/3 through ALK4, ALK5, and ALK7 [11, 297]. BMPs and their receptors are found to be expressed in mammalian female reproductive system, including oocytes [202, 260]. BMP4 and BMP7 were detected in theca/stromal cells in both rat and human ovaries. BMP6 was detected in both granulosa cells and oocytes, while BMP15 was detected exclusively in oocytes [202, 243, 298].

The processes of COC expansion and hyaluronan synthesis are stimulated by the signaling of gonadotropins and oocyte secreted cumulus expansion-enabling factors. In animal studies, TGF-β family members, especially GDF9 [207, 292, 299] and BMP15 [110, 206, 226], have been shown as prime cumulus expansion-enabling factors candidates to regulate COC expansion and the COC expansion-related genes. It has been reported that BMPs play similar roles in steroidogenesis [278, 298, 300], cell apoptosis [301, 302] and cell-cell connections [263, 303] in granulosa cells. Interestingly, unlike BMP15, exogenous treatment with BMP2 or BMP4 failed to affect COC expansion in *in vitro* maturation culture of bovine COCs [241]. Given the fact that the COC matrix is a hyaluronan-rich extracellular matrix, hyaluronan has been reported as the major structural element as backbone, covalently binding to kinds of proteoglycans and other hyaluronan-associated proteins, although the structural roles of these binding proteins on construction and regulation of the COC matrix has not been identified [51, 54, 304]. Until now, the roles of any BMP in hyaluronan production in human ovaries have not been defined. Moreover, the comparative effects of BMPs on COC expansion or COC expansion-related gene expression have not been studied in any species. Therefore, in this study, we aimed to examine

the comparative effects of different BMPs: BMP4, BMP6, BMP7, and BMP15, on hyaluronan biosynthesis and investigated the underlying mechanisms in human granulosa cells.

4.2 Materials and methods

4.2.1 Culture of primary and immortalized human granulosa cells

Primary human granulosa cells were obtained with informed patient consent following approval from the University of British Columbia Research Ethics Board. Follicular aspirates were obtained from 6 women undergoing *in vitro* fertilization, and the controlled ovarian stimulation protocol was as previously described [263]. Granulosa cells were purified by density centrifugation from follicular aspirates as previously described [305]. Briefly, each patient's follicular aspirate was centrifuged at 400 × g for 20 min and the cell pellet was resuspended in 4 ml phenol-red free DMEM/F12 medium (Invitrogen, Life Technologies) containing 10% charcoal/dextran-treated fetal bovine serum (Hyclone, GE Healthcare Life Sciences), and 1× Antibiotic-Antimycotic (Gibco, Life Technologies) and 1× Gluta MAX (Gibco, Life Technologies). Cell suspensions were layered on 8 ml Ficoll-Paque Plus (GE Healthcare Life Sciences) and centrifuged at 600 × g for 20 min. The granulosa cell layer was removed from the Ficoll-Paque column, washed with medium and resuspended in 5 ml medium. The nontumorigenic immortalized human granulosa cell line (SVOG) was previously produced by transfecting human granulosa cells with the SV40 large T antigen [265]. SVOG cells were passaged by using trypsin-EDTA solution (0.05% trypsin, 0.5mM EDTA, Invitrogen), when they reached 90% confluence. All cells were counted with a hemocytometer and cell viability was assessed by Trypan blue (0.04%) exclusion. Primary granulosa cells were seeded (1-2 × 10^5 per well) in 12-well plates for RNA, and seeded (2-4 × 10^5 per well) in 6-well plates for protein,

while SVOG cells were seeded (2-4 × 10^5 per well) in 6-well plates. Unless otherwise indicated, cells were pre-cultured for 24 hours and then maintained in serum-free medium for 24 hours prior to treatment. Culture medium was changed every other day in all experiments and the cells were cultured in a humidified atmosphere containing 5% CO_2 and 95% air at 37°C.

4.2.2 Antibodies and reagents

Polyclonal goat anti-HAS2, polyclonal rabbit anti-SMAD1/5/8, monoclonal mouse anti-α-Tubulin and horseradish peroxidase-conjugated donkey anti-goat IgG was were obtained from Santa Cruz Biotechnology. Polyclonal rabbit anti-phospho-SMAD1/5/8 ($Ser^{463/465}$, $Ser^{463/465}$, and $Ser^{426/428}$, respectively), monoclonal rabbit phospho-SMAD2 ($Ser^{465/467}$), monoclonal mouse SMAD2, monoclonal rabbit phospho-SMAD3 ($Ser^{423/425}$), monoclonal rabbit antibody SMAD3, and polyclonal rabbit anti-SMAD4 were obtained from Cell Signaling Technology. Horseradish peroxidase-conjugated goat anti-rabbit and goat anti-mouse IgG were obtained from Bio-Rad Laboratories. Recombinant human BMP4, recombinant human BMP6, recombinant human BMP7, recombinant human BMP15, dorsomorphin dihydrochloride and DMH1 were obtained from R&D Systems. SB-431542 was obtained from Sigma.

4.2.3 RT-qPCR

Total RNA was extracted with TRIzol Reagent (Invitrogen, Life Technologies) and 2 μg was reverse-transcribed into first-strand cDNA with random primers and MMLV reverse transcriptase (Promega). SYBR Green or TaqMan RT-qPCR was performed on an Applied Biosystems 7300 Real-Time PCR System equipped with 96-well optical reaction plates. Each 20 μl SYBR Green RT-qPCR reaction contained 1× SYBR Green PCR Master Mix (Applied

Biosystems), 20 ng cDNA and 250 nM of each specific primer. The primers used were: HAS2, 5′-GCCTCATCTGTGGAGATGGT-3' (sense) and 5′-TCCCAGAGGTCCACTAATGC-3' (antisense); SMAD2 (SMAD family member 2), 5′-GCCTTTACAGCTTCTCTGAACAA-3' (sense) and 5′-ATGTGGCAATCCTTTTCG AT-3' (antisense); SMAD3 (SMAD family member 3), 5′-CCCCAGCACATAACTTGG-3' (sense) and 5′-AGGAGATGGAGCACCAGAAG-3' (antisense); SMAD4 (SMAD family member 4), 5′-TGGCCCAGGATCACCAGAAG-3' (sense) and 5′-CATCAACACCAATTCCAGCA-3' (antisense); and GAPDH (glyceraldehyde-3-phosphate dehydrogenase), 5′-GAGTCAACGGATTTGGTCGT-3' (sense) and 5′-GACAAGCTTCCCGTTCTCAG-3' (antisense). Alternatively, TaqMan gene expression assays for HAS1, HAS3 and GAPDH (Hs00987418_m1, Hs00193436_m1 and Hs02758991_g1, respectively; Applied Biosystems) were performed on corresponding cDNA samples. Each 20 µl TaqMan RT-qPCR reaction contained 1 × TaqMan Gene Expression Master Mix (Applied Biosystems), 1 × specific TaqMan gene expression assay, and 20 ng cDNA. Three separate experiments were performed on different cultures and each sample was assayed in duplicate. A mean value was used for the determination of mRNA levels by the comparative Cq method ($2^{-\Delta\Delta Cq}$) with GAPDH as the reference gene.

4.2.4 Western blot analysis

Cells were seeded, treated as described, and lysed with cell lysis buffer (Cell Signaling Technology) containing protease inhibitor cocktail (Sigma-Aldrich). Extracts were centrifuged at 20,000 x g for 10 min at 4°C to remove cellular debris, and protein concentrations were quantified using the DC Protein Assay (Bio-Rad Laboratories). Equal amounts of protein (50 µg) were separated by 10% SDS-PAGE and transferred to polyvinylidene fluoride membranes. After

blocking for 1 hour with 5% nonfat dried milk in tris-buffered saline containing 0.1% Tween 20 (TBST), membranes were incubated overnight at 4°C with primary antibodies diluted 1:1000 in TBST with 5% nonfat dried milk. After washing, membranes were incubated for 1 hour with appropriate peroxidase-conjugated secondary antibody diluted 1:5000 in TBST with 5% nonfat dried milk. Immunoreactive bands were detected using enhanced chemiluminescence reagent or SuperSignal West Femto Chemiluminescence Substrate (Pierce, Thermo Scientific), followed by exposure to CL-XPosure film (Thermo Scientific). Membranes were stripped with stripping buffer (50 mM Tris-HCl pH 7.6, 10mmol/l β-mercaptoethanol and 1% SDS) at 50°C for 30 min and reprobed with anti-α-Tubulin, anti-SMAD2, anti-SMAD3, or anti-SMAD1/5/8 as a loading control.

4.2.5 siRNA transfection

To knockdown endogenous SMAD2, SMAD3 or SMAD4, cells were seeded (0.5-1 × 10^5 per well in 6-well plates) and transfected the day after seeding with 25 nM ON-TARGET*plus*SMART*pool* siRNAs targeting human SMAD2, SMAD3 or SMAD4 (Dharmacon, GE Healthcare Life Sciences) using Lipofectamine RNAiMAX (Invitrogen, Life Technologies) according to the manufacturer's instructions. ON-TARGET*plus*non-targeting control*pool* siRNA (25 nM) was used as a transfection control in all experiments. Knockdown efficiency was confirmed by RT-qPCR.

4.2.6 Measurement of hyaluronan

Cells were seeded treated as described, and conditioned medium was stored at -80°C until assayed. Hyaluronan accumulation in conditioned medium was measured as the

manufacturer's instructions using a quantitative sandwich hyaluronan enzyme immunoassay kit (detection limit, 0.068 ng/ml; R&D Systems). All samples were measured in duplicate and normalized to total cellular protein content.

4.2.7 Statistical analysis

Results are presented as the mean ± SEM of at least three independent experiments performed on separate cultures. PRISM software (GraphPad Software Inc.) was used to perform one-way ANOVA followed by Tukey's test for multiple comparisons of means, and two-tailed paired t-test for two-group comparison of means between control and specific BMP treatment groups. Means considered statistically different if $P < 0.05$ are indicated by different letters for multiple comparisons, and by * symbol for each two-group comparison by t-test.

4.3 Results

4.3.1 BMPs show differential induction of HAS2 expression in SVOG cells

To examine whether BMP4 affects HASs expression in human granulosa cells, SVOG immortalized human granulosa cells were treated with recombinant human BMP4 with 30 ng/ml for 1, 3, 6, 12, 24 hours. RT-qPCR and Western blot results showed that BMP4 treatment up-regulated HAS2 mRNA and protein levels starting at 3 hours (Figure 4-1A and 4-1B). Moreover, treatment with BMP4 with varying concentration (1, 10, 30 or 100 ng/ml) for 3 hours, up-regulated HAS2 mRNA in a concentration-dependent manner (Figure 4-1C), but had no effects on HAS3 mRNA (Figure 4-1D). HAS1 mRNA was not detectable by RT-qPCR in SVOG cells.

Next we compared the effects of 50 ng/ml BMP4, BMP6, BMP7 or BMP15 on HAS2 mRNA levels (3 hours) in SVOG human granulosa cells. RT-qPCR results showed that all of

tested BMPs increased HAS2 mRNA levels (symbol * shows a significant difference between control and specific BMP treatment groups by two-tailed paired t-test statistical analysis) with notable differential effects among different BMPs (different letters show significant difference among all groups multiple comparisons by one-way ANOVA followed by Tukey's test statistical analysis) (Figure 4-1E). BMP4 and BMP7 showed more pronounced effects (4-5 fold) than BMP6 and BMP15 (1.5-2 fold) on HAS2 mRNA increases.

4.3.2 BMPs show differential activation of noncanonical SMAD2/3 signaling in SVOG immortalized human granulosa cells

Based on the differential effects on HAS2 levels by BMPs, we compared their effects on activation of canonical and noncanonical SMAD signaling in human granulosa cells. SVOG cells were treated with BMP4, BMP6, BMP7 or BMP15 with 50 ng/ml for 30 minutes. All of tested BMPs activated canonical SMAD1/5/8 signaling with no significant different manner (Figure 4-2A). By contrast, these BMPs showed differential activation of noncanonical SMAD2 and SMAD3 phosphorylation: BMP4 and BMP7 significantly activated SMAD2 and SMAD3, while BMP6 and BMP15 had no effects on SMAD2 phosphorylation and induced SMAD3 phosphorylation very slightly (symbol * shows significant difference between control and specific BMP treatment groups by two-tailed paired t-test statistical analysis) (Figure 4-2B and 4-2C).

4.3.3 The stimulatory effects of BMP4 on HAS2 expression are partially reversed by SB-431542, which attenuates BMP4-induced noncanonical SMAD2/3 signaling without altering canonical SMAD1/5/8 signaling in SVOG cells.

Since TGF-β type I receptors (ALK1 to 7) primarily determine the specificity of SMAD signaling activation, we used three selective inhibitors to evaluate which ALK(s) is involved in the effects of BMP4 on up-regulation of HAS2, as well as the activation of canonical SMAD1/5/8 and noncanonical SMAD2/3 in human granulosa cells. These inhibitors are dorsomorphin, a specific inhibitor of ALK2/3/6 [266]; DMH1, a specific inhibitor of ALK2/3 [306]; and SB-431542, a specific inhibitor of ALK4, ALK5, and ALK7 [268]. We first treated SVOG cells with 30 ng/ml of BMP4 for 30 minutes in the presence or absence of dorsomorphin (10 μM), DMH1 (0.25 μM), or SB-431542 (10 μM) to evaluate the specific ALK(s) involved in activation of SMAD1/5/8 or SMAD2/3 signaling by BMP4. As shown in Figure 4-3A-C, Pre-treatment with dorsomorphin totally blocked the activation of both SMAD1/5/8 and SMAD2/3 by BMP4; pre-treatment with DMH1 mostly reversed the activation of both SMAD1/5/8 and SMAD2/3 by BMP4 (not significant different from control with DMH1 treatment alone); pre-treatment with SB-431542 blocked the activation of SMAD2 and partially reversed the activation of SMAD3 without altering the activation of SMAD1/5/8 by BMP4. These results suggest that ALK4/5/7 is only involved in noncanonical SMAD2/3 activation by BMP4.

Next, we treated SVOG cells with 30 ng/ml of BMP4 for 3 hours after pre-treatment with dorsomorphin, DMH1, or SB-431542. The stimulatory effects of BMP4 on HAS2 mRNA were abolished by pre-treatment with either dorsomorphin or DMH1, and partially reversed by pre-treatment with SB-431542 (Figure 4-3D). Interestingly, treatment with DMH1 alone increased the basal levels of HAS2 mRNA by 20%-30%. These results suggest that ALK4/5/7 is also

involved in the stimulatory effects of BMP4 on HAS2 in human granulosa cells. Taken together, these results indicate that BMP4 may stimulate HAS2 through noncanonical SMAD2/3 signaling, with or without canonical SMAD1/5/8 signaling in human granulosa cells.

4.3.4 Noncanonical SMAD2/3 signaling is required for BMP4- and BMP15-induced up-regulation of HAS2 in SVOG cells

To further investigate whether noncanonical SMAD2/3 singling is required for the stimulatory effects of HAS2 by BMPs, SVOG cells were pre-treated for 72 hour with siRNA targeting SMAD2, SMAD3, SMAD4 or both SMAD2 and SMAD3, treated for a further 3 hours with 30 ng/ml BMP4, or 100 ng/ml BMP15. As shown in Figure 4-4A-C, the mRNA levels of the targeted SMAD were significantly reduced by each siRNA transfection. The stimulatory effects of BMP4 on HAS2 mRNA levels were totally blocked by knockdown of SMAD4, partially reversed by knockdown of SMAD3, but not affected by knockdown of SMAD2 (Figure 4-4D). Interestingly, knockdown of both SMAD2 and 3 slightly increased the basal HAS2 mRNA levels (not significant different), but attenuated BMP4-induced HAS2 mRNA levels (Figure 4-4E). Similarly, the effects of BMP15-stimulated HAS2 mRNA were reversed by SMAD3 knockdown, blocked by both SMAD2 and 3 knockdown or SMAD4 knockdown (Figure 4-4F). These results indicate that BMP4 and BMP15 increase HAS2 mRNA through noncanonical SMAD2/3 signaling.

4.3.5 BMPs show differential induction of hyaluronan production in SVOG cells

Having demonstrated the differential induction of HAS2 expression by BMPs, we next sought to confirm the effects of BMPs on hyaluronan production in human granulosa cells. First

we examined the time-dependent effects of BMP4 on hyaluronan production in conditional medium using enzyme immunoassay followed treatment of SVOG cells with BMP4 30 ng/ml for 6, 12 and 24 hours. ELISA results showed that BMP4 up-regulated hyaluronan production by more than 2 fold starting at 24 hours (Figure 4-5A). Next, we compared the effects of BMP4, BMP6, BMP7, and BMP15 on hyaluronan production by treatment SVOG cells with 100 ng/ml of each BMP for 24 hours. Consistent with the findings for HAS2 mRNA expression, ELISA results showed that all of tested BMPs induced hyaluronan production (symbol * shows a significant difference between control and specific BMP treatment groups by two-tailed paired t-test statistical analysis) with notable differential effects among different BMPs (different letters show significant difference among all groups multiple comparisons by one-way ANOVA followed by Tukey's test statistical analysis) (Figure 4-5B).

Based on the results that the stimulatory effects of BMP4 on HAS2 mRNA expression and noncanonical SMAD2/3 activation were SB-431542 sensitive, we investigated whether the TGF-β type I receptors inhibitors regulate the effects of BMP4 on hyaluronan production. As shown in Figure 4-5C, the stimulatory effects on hyaluronan production by treatment with BMP4 30 ng/ml were blocked by dorsomorphin, DMH1 or SB-431542.

4.3.6 BMPs induce versican expression in SVOG cells

To examine the effects of BMPs on versican expression, the hyaluronan-binding protein in the COC matrix, SVOG cells were treated with BMP4 (30 ng/ml) for 3, 6, 12 and 24 hours. RT-qPCR results showed that BMP4 treatment up-regulated versican mRNA levels at 24 hours (Fig. 4-6A). Treatment with BMP4, BMP6, BMP7, and BMP15 with 50 ng/ml for 24 hours, RT-qPCR results showed that all of tested BMPs induced versican mRNA levels (symbol * shows a

significant difference between control and specific BMP treatment groups by two-tailed paired t-test statistical analysis) with notable differential effects among different BMPs (different letters show significant difference among all groups multiple comparisons by one-way ANOVA followed by Tukey's test statistical analysis) (Figure 4-6B).

4.3.7 BMP4 activates noncanonical SMAD2/3 signaling and stimulates HAS2 expression in primary human granulosa cells

Given the reports that BMP2-induced noncanonical SMAD2/3 activation preferred to occur in embryonic or transformed cell lines [140, 141], and to rule out the possibility that the noncanonical activation of SMAD2/3 by BMPs came from the immortalized transfection, we investigated the effects of BMP4 on SMAD signaling activation in primary granulosa cells from three different samples. Treatment with BMP4 (50 ng/ml) for 30 minutes activated phosphorylation of SMAD2 (Figure 4-7A), SMAD3 (Figure 4-7B), and SMAD1/5/8 (Figure 4-7C) in primary human granulosa cells from all three samples. Moreover, BMP4 stimulated HAS2 mRNA in primary human granulosa cells from another three different samples. The mRNA levels of HAS1 or HAS3 were too low to be detectable (> 15 cycles lower than GAPDH). These results suggest that it is a specific character for BMP4 to stimulate a noncanonical SMAD2/3 signaling activation and exert the functions to stimulate HAS2 in human granulosa cells.

4.4 Discussion

Successful fertilization is dependent upon proper COC expansion with a fertilized oocyte. Formation of the extracellular matrix between cumulus cells is stimulated by gonadotropins and cumulus expansion-enabling factors. Since fewer LH receptors expressed in cumulus cells and

oocytes [70], LH stimulates the expression of EGF-like growth factors, including amphiregulin, betacellulin and epiregulin, and triggers EGF signaling to activate COC expansion-related gene expression [71, 72]. Increasing numbers of studies indicate that TGF-β superfamily members, including BMPs, are candidate cumulus expansion-enabling factor due to their ability to regulate COC expansion and COC expansion-related genes expression. Homozygous null female mice of either *Bmp15* [201] or *Bmp6* [229] are subfertile with a portion exhibiting decreased ovulation (33% and 22% lower, respectively) with impaired cumulus cell function, including a small cell number [201], or down-regulated COC transcripts [229]. In COCs *in vitro* culture studies, BMP15 stimulated COC expansion, and COC expansion related genes, including HAS2, in mouse [110], cow [226] and pig [206], while BMP2 or BMP4 had no effects on COC expansion in cow [241]. Moreover, BMP15 has been shown to stimulate mRNA levels of EGF-like growth factors: amphiregulin and epiregulin in bovine cumulus cells [226], and amphiregulin, betacellulin and epiregulin in mouse COCs [110]. In the present study, we first showed that in human granulosa cells, BMP4, BMP6, BMP7 and BMP15 all stimulate HAS2 expression and hyaluronan production as well as the expression of the hyaluronan-binding protein versican. Moreover, the differential activation of noncanonical SMAD2/3 signaling by these BMPs causes disproportionate induction of hyaluronan production.

These results are in keeping with a recent but expanding body of literature that challenges the common idea that BMPs exert their functions through canonical SMAD1/5/8, but not SMAD2/3. In B16 melanoma cells, BMP2 was found to phosphorylate SMAD2 and up-regulate the SMAD2/3 target gene, plasminogen activator inhibitor-1 [307]. Similarly, in murine gonadotrope-like LβT2 cells, BMP2 phosphorylated SMAD2, activated SMAD3-dependent reporter, and stimulated *Fshb* transcription via SMAD3 [141]. Moreover, BMP2 was also

reported to phosphorylate SMAD2/3 in many (89%; 25/28) cancer cell lines and some (33%; 4/12) nontransformed cell lines [140]. In addition, BMP2, BMP4 and BMP7 have been shown to activate SMAD2/3 in normal ovarian surface epithelial, and human pancreatic and breast cancer cells [140]. Recombinant human BMP15, but not mouse BMP15, was found to stimulate mouse COC expansion, and mildly phosphorylate SMAD2/3 in mouse granulosa cells and human granulosa tumor cells [207]. Previous studies have implicated type I and II receptor expression levels as a contributing factor for the differential activation of noncanonical SMAD2/3 signaling by BMP2 between some cell lines. In particular, cell lines with higher receptor expression levels tended to exhibit noncanonical SMAD2/3 activation in response to BMP2, as well as noncanonical SMAD1/5/8 activation in response to TGF-β [307]. It remains to be determined to what degree receptor expression levels contribute to the differential activation of noncanonical SMAD2/3 signaling in human granulosa cells (e.g. mural *vs.* cumulus granulosa cells). However, my study clearly shows that BMPs can exert differential effects on noncanonical SMAD2/3 activation within a single cell line expressing a fixed repertoire of receptors. Thus, differences in receptor binding properties between different BMPs are also likely to contribute to the differential activation of noncanonical SMAD2/3.

My inhibitor results suggest that ALK2/3/6 (mostly ALK2/3) participate in BMP4-stimulated canonical SMAD1/5/8 and noncanonical SMAD2/3 activation whereas ALK4/5/7 are only involved in noncanonical SMAD2/3 activation. It is commonly held that the L45 loop (a region between kinase subdomains IV and V) of TGF-β type I receptors is critical for determining the activation of either SMAD2/3 or SMAD1/5/8 signaling. Interestingly, 5 of the 9 amino acid residues in the L45 loop of ALK4/5/7 are conserved in that of ALK3/6, whereas only 1 of 9 amino acid residues are conserved in ALK2 [308, 309]. Moreover, the activation of

SMAD3 in LβT2 cells and SMAD2 in B16 cells by BMP2 (which shares >90% amino acid sequence homology to BMP4) have been shown to be mediated primarily by ALK3 [141, 307]. Therefore, I believe that the BMP4-induced activation of SMAD2/3 in human granulosa cells is likely to be partly mediated by ALK3 rather than ALK2. In addition to ALK3, BMP4-induced activation of SMAD2/3 requires ALK4/5/7 given that SB-431542 blocked the activation of SMAD2 and partially reversed the activation of SMAD3. These effects are most likely mediated by ALK4/5 since ALK7 is primarily expressed in adipose tissue [310] and is virtually undetectable in human granulosa cells (my unpublished data). Hence, ALK4/5 might combine with ALK3 forming heteromeric receptor complexes, including ALK3/4, ALK3/5 or both [140, 311].

Interestingly, previous studies have shown that BMPs could activate SMAD2 and SMAD3 through different ALK(s) in specific cell types. In LβT2 cells, the mutant ALK3 (ALK3-6SA) blocked BMP2-induced SMAD3 activation but not SMAD2 phosphorylation [141]. By contrast, another study showed that BMP2 activated SMAD2 phosphorylation through ALK3/5 while SMAD3 phosphorylation occurs through ALK3/5 or ALK6/7 [140]. Moreover, in B16 cells, BMP2-induced phosphorylation of SMAD2 was significantly reversed by ALK3 or ALK4 knockdown [307]. Given the results that SB-431542 totally blocked BMP4-induced SMAD2 phosphorylation while partially reversed SMAD3 phosphorylation, we suggest that in human granulosa cells BMP4 activated SMAD2 phosphorylation through the heteromeric type I receptors of ALK3/4 or ALK3/5, while activated SMAD3 phosphorylation through both ALK3 only and the heteromeric receptors of ALK3/4 or ALK3/5. Interestingly, BMP2 was showed that to activate SMAD2 phosphorylation through TGFBR2, while activate SMAD3 phosphorylation

through BMPR2 [140]. These results indicate that TGF-β type II receptors might be involved in noncanonical signaling induced by BMPs.

In the present study, we found that BMP-induced noncanonical SMAD2/3 signaling activation contributed to the induction of hyaluronan production in human granulosa cells. Accumulated evidence from animal studies suggests that SMAD2/3 signaling is essential to COC expansion. First, granulosa cell-specific knockout mouse models of SMAD2, SMAD3 or both, showed that SMAD2 and SMAD3 exerted redundant roles on COC expansion: only the *Smad2*/*Smad3* double mutant female mice had a minimal expansion of cumulus cells [256]. Second, the SMAD2/3 activation TGF-β superfamily members, such as GDF9, TGF-β1 and activins, all regulated COC expansion and stimulated the expression of COC expansion-related genes, including HAS2 [119, 256]. Third, the inhibitor (SB-431542) of ALK4/5/7 that mainly activates SMAD2/3 significantly reduced gonadotropin-induced murine COC expansion [119, 312]. Moreover, the specific inhibitor of SMAD3 (SIS3) [313] had stronger effects on preventing FSH-induced porcine COC expansion than SB-431542 [314]. These inhibitors significantly reduced FSH- or EGF- stimulated hyaluronan synthesis and incorporation within the COC matrix [314]. By contrast, dorsomorphin was shown to reduce FSH-induced cumulus expansion index in pig but not in mouse [312]. In a study of mouse COC *in vitro* cultures, recombinant human BMP15 activated noncanonical SMAD2/3 and stimulated COC expansion as well as HAS2 mRNA. The up-regulation of HAS2 mRNA by BMP15 was abolished by LDN-193189 (inhibitor of ALK2/3/6) [207]. Our findings that BMPs induced hyaluronan production via activation of noncanonical SMAD2/3 in human granulosa cells lead to a new concept that BMPs may be similar to GDF9 and TGF-β to regulate COC expansion through SMAD2/3 in the ovaries.

In the present study, HAS2 and HAS3 mRNAs were detected in SVOG cells while only HAS2 mRNA was detected in primary human granulosa cells. Regarding the time-course results (< 3 hours) and the SMAD binding-sites located on *HAS2* promoter [315], we suggested that BMPs regulated HAS2 expression through directly binding to the *HAS2* promoter via SMAD2/3 in human granulosa cells. Similarly, BMP15 was found to increase HAS2 mRNA levels at 5 hours in mouse [207], and at 12 but not 22 hours in bovine cumulus cells [205, 316]. Our previous study showed that BMP4 and BMP7 down-regulated StAR expression through canonical SMAD1/5/8, but not noncanonical SMAD2/3 (Figure 3-9). Interestingly, another study showed that BMP15 exerted different effects in rat granulosa cells through different signaling (SMAD-dependent or SMAD-independent signaling) [317]. We therefore suggest that BMPs exert different functions through different SMAD signaling (canonical or noncanonical) in human granulosa cells.

In summary, the present study demonstrates that BMP4, BMP6, BMP7 and BMP15 all up-regulate HAS2 expression and hyaluronan production as well as the expression of versican, the hyaluronan binding protein, in human granulosa cells. Differential activation of noncanonical SMAD2/3 signaling by BMPs causes disproportionate induction of HAS2 expression and hyaluronan production in human granulosa cells. Moreover, the stimulatory effects of both noncanonical SMAD2/3 activation and HAS2 expression by BMPs are attenuated by SB-431542, which suggests ALK4/5/7 is involved in the effects of BMPs. These findings provide important insight into the functional roles of BMPs on COC expansion through a noncanonical SMAD signaling pathway.

4.5 Figures

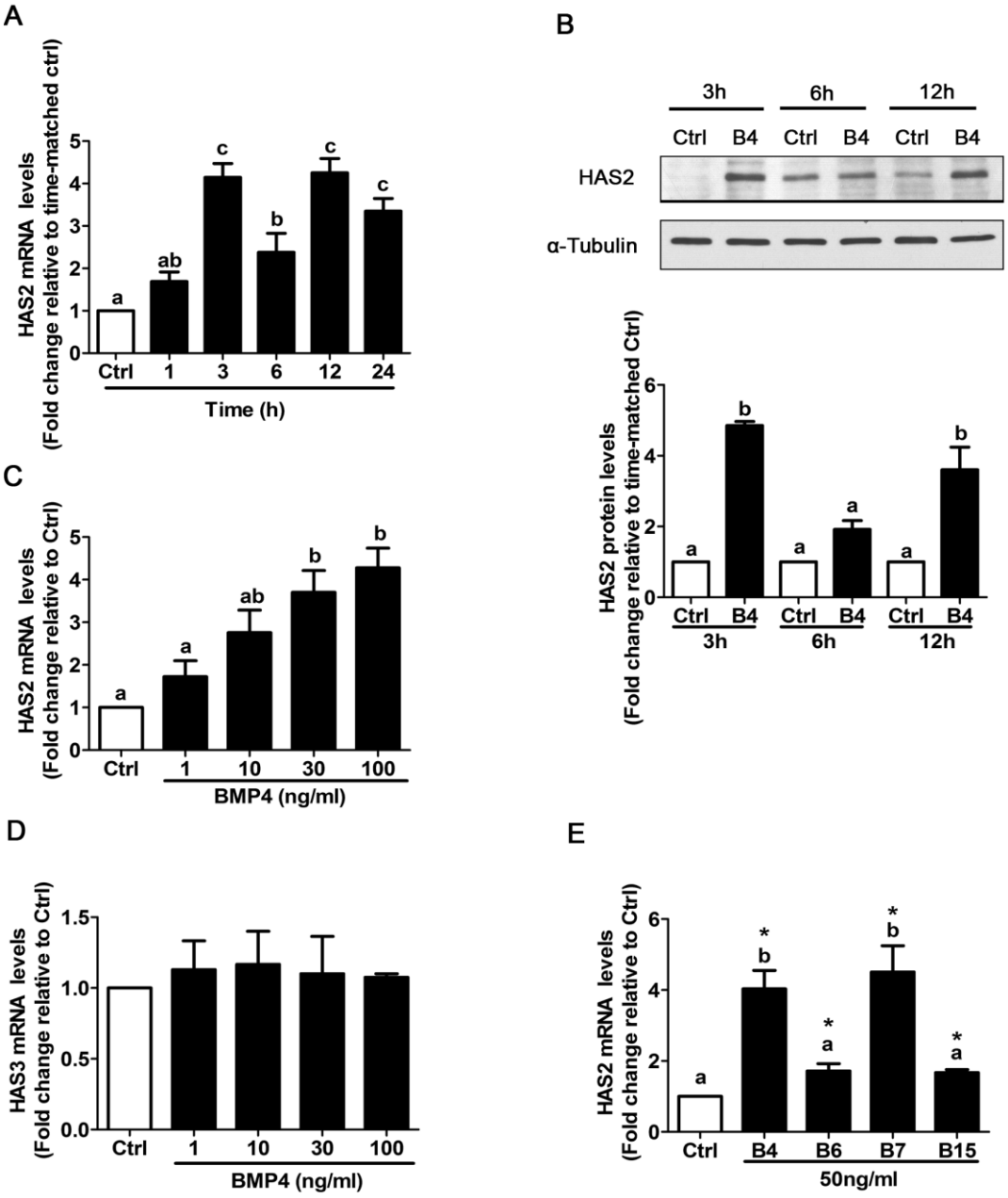
A
HAS2 mRNA levels
(Fold change relative to time-matched ctrl)
Ctrl 1 3 6 12 24
Time (h)
B
3h 6h 12h
Ctrl B4 Ctrl B4 Ctrl B4
HAS2
α-Tubulin
HAS2 protein levels
(Fold change relative to time-matched Ctrl)
C
HAS2 mRNA levels
(Fold change relative to Ctrl)
Ctrl 1 10 30 100
BMP4 (ng/ml)
D
HAS3 mRNA levels
(Fold change relative to Ctrl)
Ctrl 1 10 30 100
BMP4 (ng/ml)
E
HAS2 mRNA levels
(Fold change relative to Ctrl)
Ctrl B4 B6 B7 B15
50ng/ml

Figure 4-1 BMPs show differential induction of HAS2 expression in SVOG immortalized human granulosa cells.

Cells were treated with vehicle control (Ctrl) or 30 ng/ml of BMP4 for 1h, 3h, 6h, 12h and 24 h, and the mRNA levels (A) and protein levels (B) of HAS2 were examined by RT-qPCR and Western blot, respectively. Cells were treated with vehicle control (Ctrl) or increasing concentrations (1, 10, 30 and 100 ng/ml) of BMP4 for 3 hours, and the mRNA levels of HAS1 (not detectable), HAS2 (C) and HAS3 (D) were examined by RT-qPCR. Cells were treated with vehicle control (Ctrl) or 50 ng/ml of BMP4, BMP6, BMP7 or BMP15 for 3 hours, and the HAS2 mRNA levels were examined by RT-qPCR (E). GAPDH mRNA levels and α-Tubulin protein levels were used as internal controls. Values marked by different letters are significantly different ($p<0.05$) by one-way ANOVA followed by Tukey's test, and values marked ***** are significantly different ($p<0.05$) by two-tailed paired t-test between Ctrl and each specific BMP treatment groups.

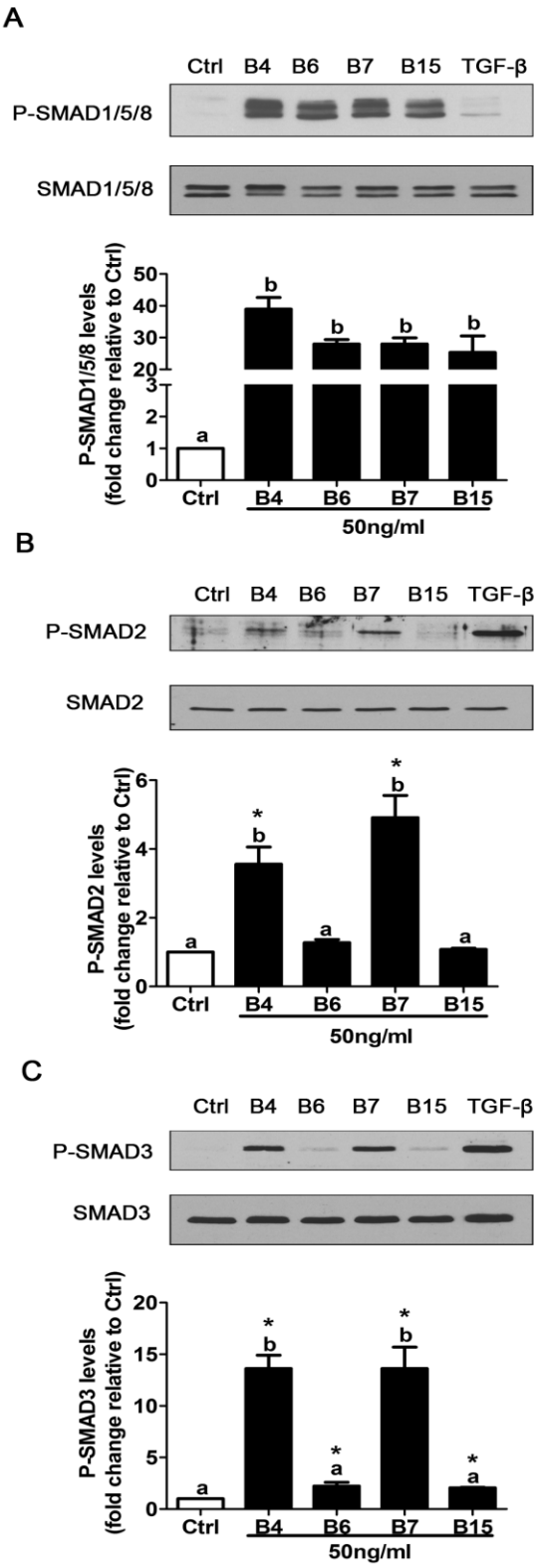
A
Ctrl B4 B6 B7 B15 TGF-β
P-SMAD1/5/8
SMAD1/5/8
P-SMAD1/5/8 levels (fold change relative to Ctrl)
50
40
30
20
3
2
1
0
a
b
b
b
b
Ctrl B4 B6 B7 B15
50ng/ml
B
Ctrl B4 B6 B7 B15 TGF-β
P-SMAD2
SMAD2
P-SMAD2 levels (fold change relative to Ctrl)
6
4
2
0
a
*b
a
*b
a
Ctrl B4 B6 B7 B15
50ng/ml
C
Ctrl B4 B6 B7 B15 TGF-β
P-SMAD3
SMAD3
P-SMAD3 levels (fold change relative to Ctrl)
20
15
10
5
0
a
*b
*a
*b
*a
Ctrl B4 B6 B7 B15
50ng/ml

Figure 4-2 BMPs show differential activation of noncanonical SMAD2/3 signaling in SVOG immortalized human granulosa cells.

Cells were treated with vehicle control (Ctrl) or 50 ng/ml of BMP4, BMP6, BMP7, BMP15 or 10 ng/ml of TGF-β for 30 minutes, and the phosphorylated and the basal levels of SMAD1/5/8 (A), SMAD2 (B) and SMAD3 (C) were determined by Western blot. Results are expressed as the mean ± SEM of at least three independent experiments. Values marked by different letters are significantly different ($p<0.05$) by one-way ANOVA followed by Tukey's test, and values marked ***** are significantly different ($p<0.05$) by two-tailed paired t-test between Ctrl and each specific BMP treatment groups.

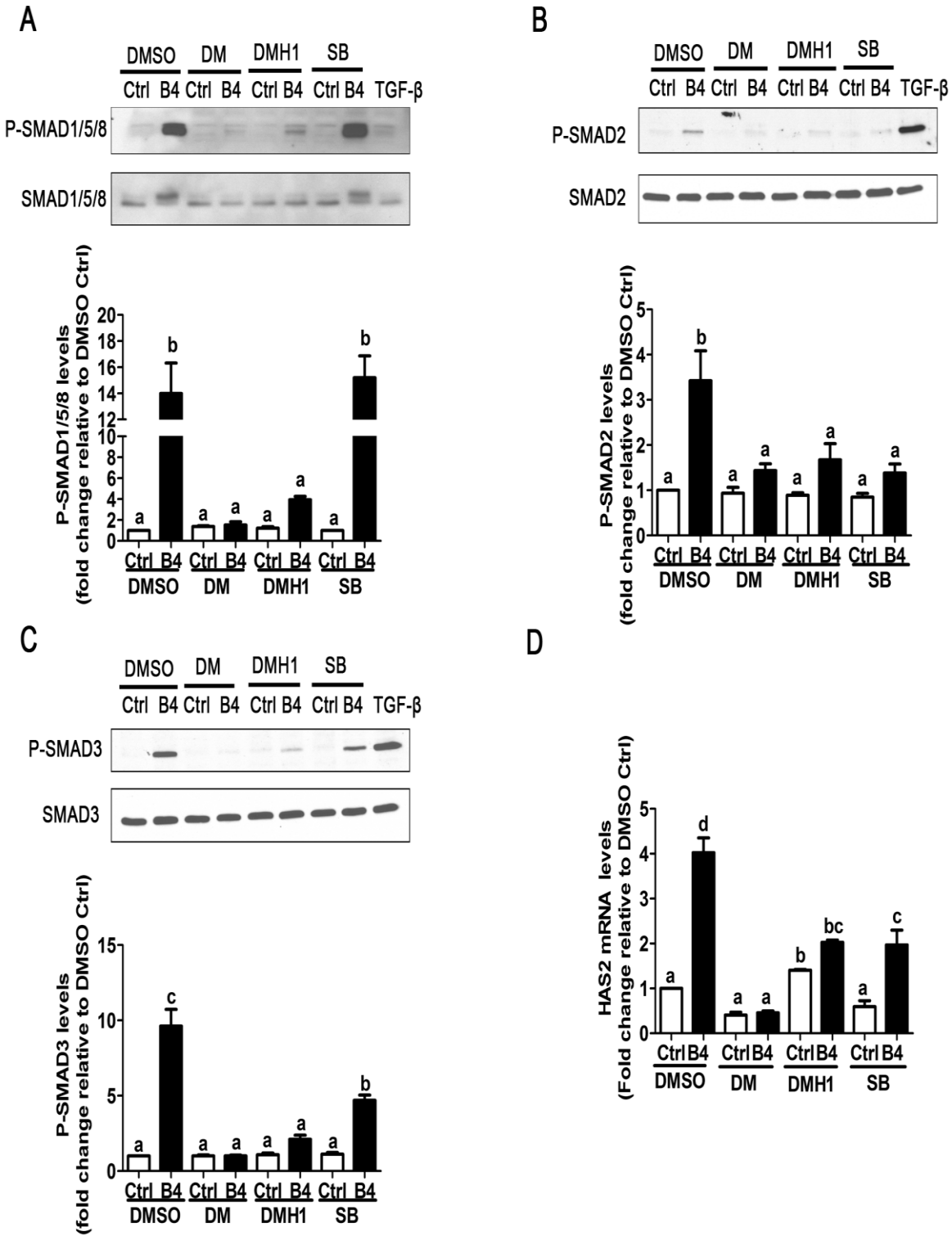
A
DMSO
DM
DMH1
SB
Ctrl B4 Ctrl B4 Ctrl B4 Ctrl B4 TGF-β
P-SMAD1/5/8
SMAD1/5/8
P-SMAD1/5/8 levels (fold change relative to DMSO Ctrl)
Ctrl B4 Ctrl B4 Ctrl B4 Ctrl B4
DMSO DM DMH1 SB
B
P-SMAD2
SMAD2
P-SMAD2 levels (fold change relative to DMSO Ctrl)
C
P-SMAD3
SMAD3
P-SMAD3 levels (fold change relative to DMSO Ctrl)
D
HAS2 mRNA levels (Fold change relative to DMSO Ctrl)

Figure 4-3 The stimulatory effects of BMP4 on HAS2 expression are partially reversed by SB-431542 that attenuates BMP4-induced noncanonical SMAD2/3 signaling without altering canonical SMAD1/5/8 signaling in SVOG cells.

SVOG cells were treated with 30 ng/ml of BMP4 in the absence (DMSO) or presence of dorsomorphin (10 μM) or DMH1 (0.25 μM) or SB- 431542 (10 μM), as well as 10 ng/ml TGF-β for 30 minutes. The phosphorylated and the basal levels of SMAD1/5/8 (A), SMAD2 (B) and SMAD3 (C) were examined by Western blot. SVOG cells were treated with 30 ng/ml of BMP4 for 3 h in the absence (DMSO) or presence of dorsomorphin (10 μM) or DMH1 (0.25 μM) or SB- 431542 (10 μM). The mRNA levels of HAS2 were examined by RT-qPCR (D). GAPDH mRNA levels were used as an internal control. Results are expressed as the mean ± SEM of at least three independent experiments and values marked by different letters are significantly different ($p<0.05$).

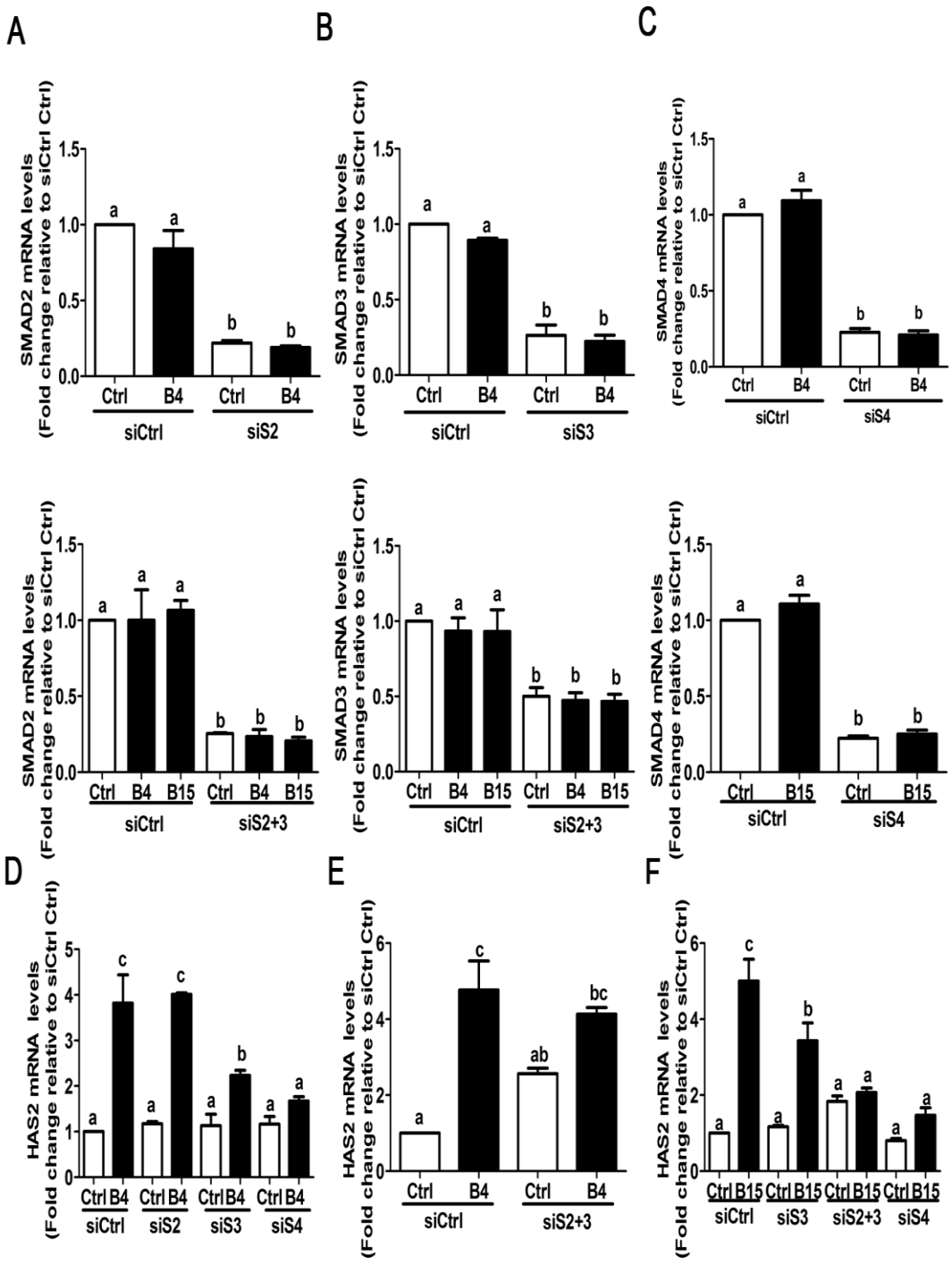
A
B
C
SMAD2 mRNA levels (Fold change relative to siCtrl Ctrl)
SMAD3 mRNA levels (Fold change relative to siCtrl Ctrl)
SMAD4 mRNA levels (Fold change relative to siCtrl Ctrl)
Ctrl B4
siCtrl siS2
siCtrl siS3
siCtrl siS4
Ctrl B4 B15
siCtrl siS2+3
Ctrl B15
siCtrl siS4
D
E
F
HAS2 mRNA levels (Fold change relative to siCtrl Ctrl)
siCtrl siS2 siS3 siS4
siCtrl siS2+3
siCtrl siS3 siS2+3 siS4

Figure 4-4 Noncanonical SMAD2/3 signaling is required for BMP4-induced up-regulation of HAS2 expression in SVOG immortalized human granulosa cells.

Cells were transfected with 25 nM control siRNA (siCtrl), SMAD2 siRNA (siS2), SMAD3 siRNA (siS3), SMAD4 siRNA (siS4) or both SMAD2 and SMAD3 siRNA (siS2+3) for 72 hours, and then treated with BMP4 (30 ng/ml), or BMP15 (100 ng/ml) for 3 hours. The mRNA levels of SMAD2 (A), SMAD3 (B), SMAD4 (C) and HAS2 (D-F) were examined by RT-PCR. GAPDH mRNA levels were used as an internal control. Results are expressed as the mean ± SEM of at least three independent experiments and values marked by different letters are significantly different ($p<0.05$).

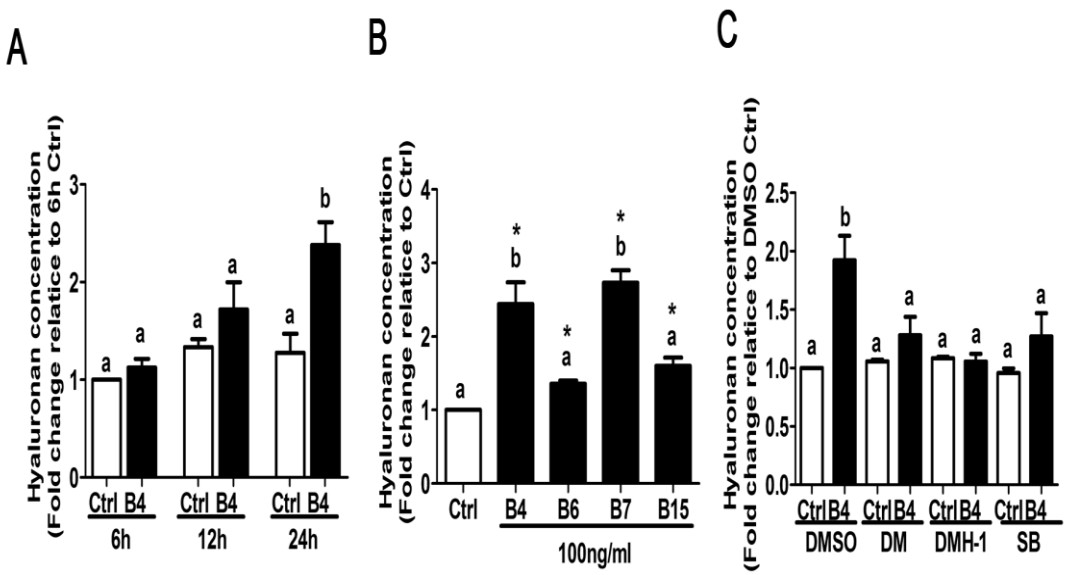

Figure 4-5 BMPs show differential induction of hyaluronan production in SVOG immortalized human granulosa cells.

Cells were treated with vehicle control (Ctrl) or 30 ng/ml of BMP4 for 6h, 12h and 24h, and the hyaluronan accumulation levels in conditional media were examined by ELISA (A). Cells were treated with vehicle control (Ctrl) or 100 ng/ml of BMP4, BMP6, BMP7 or BMP15 for 24 hours, and the hyaluronan accumulation levels in conditional media were examined by ELISA (B). Cells were treated with vehicle control (Ctrl) or 30 ng/ml of BMP4 for 24 hours in the absence (DMSO) or presence of dorsomorphin (10 μM) or DMH1 (0.25 μM) or SB- 431542 (10 μM), and the hyaluronan accumulation levels in conditional media were examined by ELISA (C). Results are expressed as the mean ± SEM of at least three independent experiments. Values marked by different letters are significantly different ($p<0.05$) by one-way ANOVA followed by Tukey's test, and values marked ***** are significantly different ($p<0.05$) by two-tailed paired t-test between Ctrl and each specific BMP treatment groups.

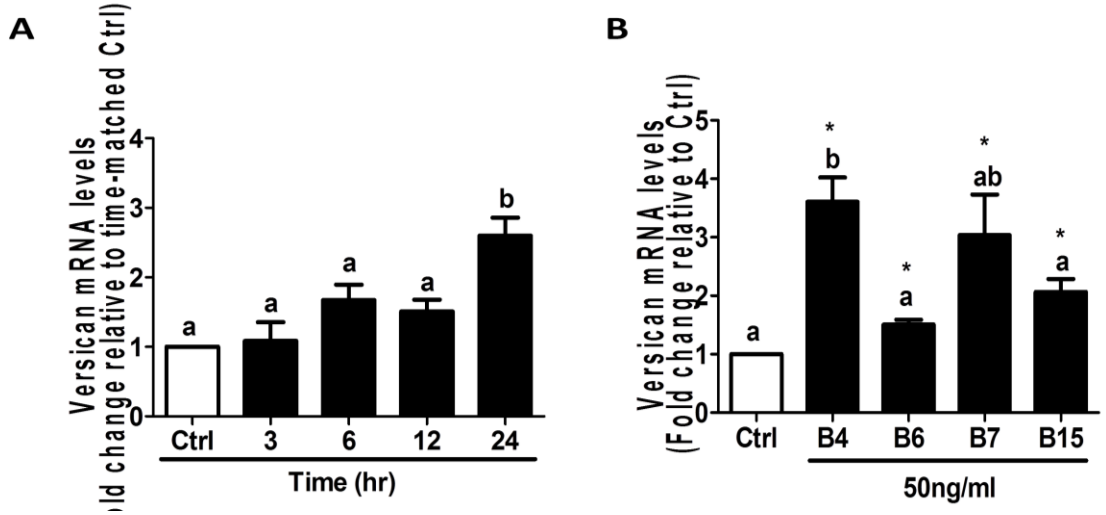

Figure 4-6 BMPs up-regulate versican expression in SVOG immortalized human granulosa cells.

Cells were treated with vehicle control (Ctrl) or 30 ng/ml of BMP4 for 1h, 3h, 6h, 12h and 24h, and the versican mRNA levels by RT-qPCR (A). Cells were treated with vehicle control (Ctrl) or 50 ng/ml of BMP4, BMP6, BMP7 or BMP15 for 3 hours, and the versican mRNA levels were examined by RT-qPCR (B). GAPDH mRNA levels were used as an internal control. Values marked by different letters are significantly different ($p<0.05$) by one-way ANOVA followed by Tukey's test, and values marked * are significantly different ($p<0.05$) by two-tailed paired t-test between Ctrl and each specific BMP treatment groups.

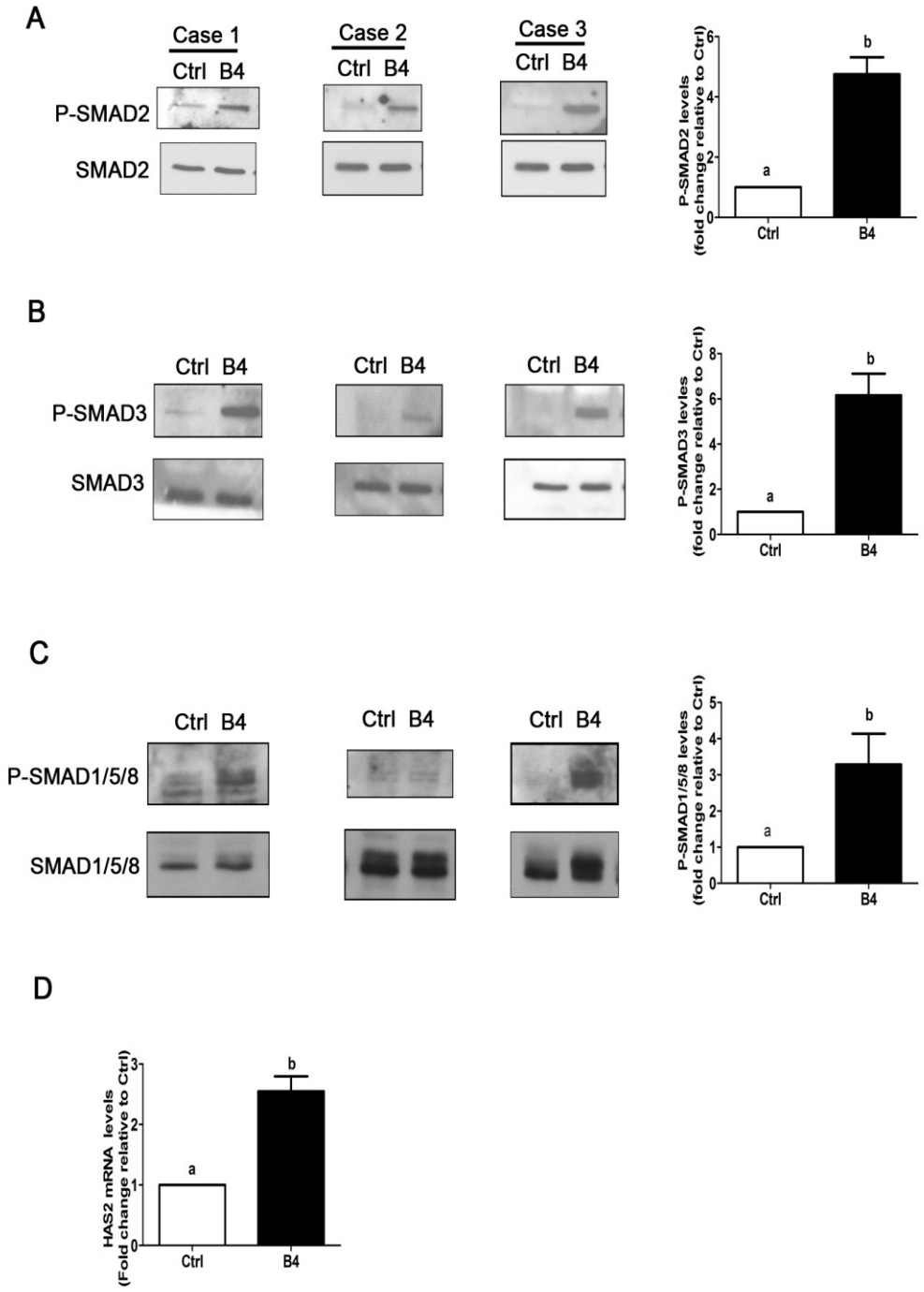
A
Case 1
Case 2
Case 3
Ctrl B4
Ctrl B4
Ctrl B4
P-SMAD2
SMAD2
P-SMAD2 levels
(fold change relative to Ctrl)
a
b
Ctrl
B4
B
Ctrl B4
Ctrl B4
Ctrl B4
P-SMAD3
SMAD3
P-SMAD3 levles
(fold change relative to Ctrl)
a
b
Ctrl
B4
C
Ctrl B4
Ctrl B4
Ctrl B4
P-SMAD1/5/8
SMAD1/5/8
P-SMAD1/5/8 levles
(fold change relative to Ctrl)
a
b
Ctrl
B4
D
HAS2 mRNA levels
(Fold change relative to Ctrl)
a
b
Ctrl
B4

Figure 4-7 BMP4 activates both noncanonical SMAD2/3 and canonical SMAD1/5/8 signaling, and stimulates HAS2 expression in primary human granulosa cells.

Primary granulosa cells were treated with vehicle control (Ctrl) or 50 ng/ml of BMP4 for 30 min, and the phosphorylated and the basal levels of SMAD2 (A), SMAD3 (B) and SMAD1/5/8 (C) were determined by Western blot. Primary granulosa cells were treated with vehicle control (Ctrl) or 50 ng/ml of BMP4 for 3 hours, the mRNA levels of HAS2 were examined by RT-qPCR (D). GAPDH mRNA levels were used as an internal control. Results are expressed as the mean ± SEM of three different cases. Values marked by different letters are significantly different ($p<0.05$) by two-tailed paired t-test.

Chapter 5: BMP4 induces ADAMTS-1 and the functions of versican proteolytic cleavage

5.1 Introduction

Ovulation, the process ends with COC discharged from the follicular wall and released into Fallopian tubes after the follicular wall bursts. The successful follicle rupture depends on the hyaluronan-rich COC matrix and the proteases attached to the matrix. ADAMTS-1, first identified as a cachexigenic tumour selective gene product and suggested to be involved in various inflammatory processes [318], is the only protease so far demonstrated to be essential for follicle rupture. As for other ADAMTS family members, ADAMTS-1 consists of four functional subunits: an amino-terminal prodomain, a catalytic domain, a disintegrin-like domain and a carboxyl-terminal ECM binding domain. The ECM binding domain includes a central thrombospondin (TSP) type 1 motif, a spacer region and the second TSP-like motifs [318, 319]. The mature ADAMTS-1 is secreted into the ECM after the excision of its prodomain by furin-related endopeptidases [56, 318, 319]. *Adamts-1* null female mice featured markedly decreased ovulation rates, decreased the number of late preantral and later stages follicles, increased trapped mature oocytes within luteinized follicles [320-322], as well as disrupted structural organization of the COC matrix [323]. These studies indicated that ADAMTS-1 played an important role in ovulation, folliculogenesis and vascularization in female ovaries.

As a proteinase, ADAMTS-1 mediates ECM remodeling during the periovulatory interval by proteolytic cleavage of versican and aggrecan [56]. Versican, also known as chondroitin sulfate proteoglycan 2 (CSPG2), is a ubiquitous hyaluronan binding proteoglycan, and is expressed in the ECM of a broad-spectrum tissues [324, 325]. There is a single versican

gene (VCAN) and four alternative splicing mRNA products. Four distinct isoforms (V0, V1, V2 and V3) share a common N-terminal hyaluronan-binding domain and a common C-terminal lectin binding domain but different glycosaminoglycan (GAG)-binding regions [326]. *Vcan* knockout mice have the identical phenotype as *Has2* null mice: dying during embryogenesis with malformation of endocardial cushion formation [287, 327]. Versican plays a critical role as a structural molecule in hyaluronan-rich ECM and strongly interacts with other ECM molecules to regulate the loose and hydrated matrix. In mouse ovaries, versican V0, V1 and V3 isoforms were detected throughout follicular development, especially in the expanded COC matrix, basement membrane and the newly formed corpora lutea [73]. Similarly, in bovine ovaries, V0 and V1 isoforms of versican were localized in the granulosa layers and basal lamina in the preovulatory follicles [328].

ADAMTS-1 expression is regulated by growth factors such as TGF-β and IL-1β [329] and hormones such as progesterone, estradiol and dihydrotestosterone (DHT) [330]. In mice, *Adamts-1* expression is induced by LH and regulated by PR in granulosa cells in the preovulatory follicles [56]. Similarly, the expression of versican in basal lamina cannot be detected during early folliculogenesis, but is dramatically increased in granulosa cells after the LH surge [331]. However, the versican mRNA levels induced by LH were not affected in either *Cox2* or *Pgr* knockout mice [73]. My previous studies found that BMPs up-regulate versican mRNA levels in human granulosa cells. Based on the fact that versican V0/V1 could be cleaved primarily by ADAMTS-1, generating an N-terminal neoepitope DPEAAE, in this study, I examined the effects of recombinant BMP4 on ADAMTS-1 expression and the functions of versican cleavage in human granulosa cells.

5.2 Materials and methods

5.2.1 Culture of primary and immortalized human granulosa cells

Primary human granulosa cells were obtained with informed patient consent following approval from the University of British Columbia Research Ethics Board. Follicular aspirates were obtained from 3 women undergoing in vitro fertilization, and the controlled ovarian stimulation protocol was as previously described [263]. Granulosa cells were purified by density centrifugation from follicular aspirates as previously described [305]. Briefly, each patient's follicular aspirate was centrifuged at 400 × g for 20 min and the cell pellet was resuspended in 4 ml phenol-red free DMEM/F12 medium (Invitrogen, Life Technologies) containing 10% charcoal/dextran-treated fetal bovine serum (Hyclone, GE Healthcare Life Sciences), and 1× Antibiotic-Antimycotic (Gibco, Life Technologies) and 1× Gluta MAX (Gibco, Life Technologies). Cell suspensions were layered on 8 ml Ficoll-Paque Plus (GE Healthcare Life Sciences) and centrifuged at 600 × g for 20 min. The granulosa cell layer was removed from the Ficoll-Paque column, washed with medium and resuspended in 5 ml medium. The nontumorigenic immortalized human granulosa cell line (SVOG) was previously produced by transfecting human granulosa cells with the SV40 large T antigen [265]. SVOG cells were passaged by using trypsin-EDTA solution (0.05% trypsin, 0.5mM EDTA, Invitrogen), when they reached 90% confluence. All cells were counted with a hemocytometer and cell viability was assessed by Trypan blue (0.04%) exclusion. Both primary granulosa cells and SVOG cells were seeded (2-4 × 10^5 per well) in 6-well plates for protein. Unless otherwise indicated, cells were pre-cultured for 24 hours and then maintained in serum-free medium for 24 hours prior to treatment. Culture medium was changed every other day in all experiments and the cells were cultured in a humidified atmosphere containing 5% CO_2 and 95% air at 37°C.

5.2.2 Antibodies and reagents

Polyclonal rabbit anti-ADAMTS-1 was obtained from Biodesign Intl. Monoclonal mouse anti-α-Tubulin was obtained from Santa Cruz Biotechnology. Polyclonal rabbit anti-Versican V1 neoepitope and polyclonal rabbit full-length Versican V0/V1 were obtained from Abcam Inc. Horseradish peroxidase-conjugated goat anti-rabbit and goat anti-mouse IgG were obtained from Bio-Rad Laboratories. Recombinant human BMP4 was obtained from R&D Systems.

5.2.3 RT-qPCR

Total RNA was extracted with TRIzol Reagent (Invitrogen, Life Technologies) and 2 μg was reverse-transcribed into first-strand cDNA with random primers and MMLV reverse transcriptase (Promega). SYBR Green RT-qPCR was performed on an Applied Biosystems 7300 Real-Time PCR System equipped with 96-well optical reaction plates. Each 20 μl SYBR Green RT-qPCR reaction contained 1× SYBR Green PCR Master Mix (Applied Biosystems), 20 ng cDNA and 250 nM of each specific primer. The primers used were: VCAN (versican), 5′-TCAGCCTACCTTGTCATTTTTCAAC-3' (sense) and 5′-CATTTGATGCGGAGAAATTCAC-3' (antisense); ADAMTS-1, 5′-GCTCATCTGCCAAGCCAAAG–3' (sense), and 5′-CTACAACCTTGGGCTGCAAAA–3' (antisense); SMAD4, 5′-TGGCCCAGGATCACCAGAAG–3' (sense), and 5′-CATCAACACCAATTCCAGCA–3' (antisense); GAPDH, 5′-GAGTCAACGGATTTGGTCGT–3' (sense), and 5′-GACAAGCTTCCCGTTCTCAG-3' (antisense). Three separate experiments were performed on different cultures and each sample was assayed in duplicate. A mean value was used for the

determination of mRNA levels by the comparative Cq method ($2^{-\Delta\Delta Cq}$) with GAPDH as the reference gene.

5.2.4 Western blot analysis

Cells were seeded, treated as described, and lysed with cell lysis buffer (Cell Signaling Technology) containing protease inhibitor cocktail (Sigma-Aldrich). Extracts were centrifuged at 20,000 x g for 10 min at 4°C to remove cellular debris, and protein concentrations were quantified using the DC Protein Assay (Bio-Rad Laboratories). Equal amounts of protein (50 µg) were separated by 10% SDS-PAGE and transferred to nitrocelluloe membranes. After blocking for 1 hour with 5% nonfat dried milk in tris-buffered saline containing 0.1% Tween 20 (TBST), membranes were incubated overnight at 4°C with primary antibodies diluted 1:1000 in TBST with 5% nonfat dried milk. After washing, membranes were incubated for 1 hour with appropriate peroxidase-conjugated secondary antibody diluted 1:5000 in TBST with 5% nonfat dried milk. Immunoreactive bands were detected using enhanced chemiluminescence reagent or SuperSignal West Femto Chemiluminescence Substrate (Pierce, Thermo Scientific), followed by exposure to CL-XPosure film (Thermo Scientific). Membranes were stripped with stripping buffer (50 mM Tris-HCl pH 7.6, 10mmol/l β-mercaptoethanol and 1% SDS) at 50°C for 30 min and reprobed with anti-α-Tubulin as a loading control.

5.2.5 siRNA transfection

To knockdown endogenous SMAD4, cells were seeded (0.5-1 × 10^5 per well in 6-well plates) and transfected the day after seeding with 25 nM ON-TARGET*plus*SMART*pool* siRNAs targeting human SMAD4 (Dharmacon, GE Healthcare Life Sciences) using Lipofectamine

RNAiMAX (Invitrogen, Life Technologies) according to the manufacturer's instructions. ON-TARGET*plus*non-targeting control*pool* siRNA (25 nM) was used as a transfection control in all experiments. To knock down endogenous ADAMTS-1, cells were transfected with 10 nM single duplex siRNAs targeting human ADAMTS-1 (Hs_ADAMTS1_1 FlexiTube siRNA (SI00093562), Qiagen, Co) using Lipofectamine RNAiMAX. The knockdown efficiency of ADAMTS-1 and SMAD4 was confirmed by RT-qPCR and Western blot.

5.2.6 Statistical analysis

Results are presented as the mean ± SEM of at least three independent experiments performed on separate cultures. PRISM software (GraphPad Software Inc.) was used to perform one-way ANOVA followed by Tukey's test for multiple comparisons of means. Means were considered statistically different if $P < 0.05$ and are indicated by different letters.

5.3 Results

5.3.1 BMP4 stimulates ADAMTS-1 protein and the cleaved versican in SVOG immortalized human granulosa cells.

To examine the effects of BMP4 on ADAMTS-1 expression and the functions of versican cleavage, SVOG cells were treated with BMP4 30 ng/ml for 3, 6, 12, 24 hours. As shown in Figure 5-1, BMP4 did not affect ADAMTS-1 mRNA levels by RT-qPCR (Figure 5-1B), but up-regulated ADAMTS-1 protein levels starting at 6 hours by Western blot (Figure 5-1A). The full-length versican protein levels were not affected by treatment with BMP4 (Figure 5-1C), while the DPEAAE, a neoepitope of proteolytic cleavage of versican by ADAMTS-1, increased at 24 hours (Figure 5-1D).

5.3.2 ADAMTS-1 is involved in BMP4-induced versican cleavage in SVOG cells.

To further examine whether ADAMTS-1 is involved in generation of neoepitope of cleaved versican in human granulosa cells, SVOG cells were pre-treated for 48 hours with siRNA targeting ADAMTS-1 (10 nM), treated for a further 24 hours with 30 ng/ml BMP4. The mRNA levels of ADAMTS-1 were reduced about 40% by siRNA transfection (Figure 5-2A). As shown in Figure 5-2B, BMP4-induced production of cleaved versican (DPEAAE) was partially reversed. These results indicate that ADAMTS-1 is involved in BMP4-induced proteolytic cleavage of versican in immortalized human granulosa cells.

5.3.3 BMP4 stimulates ADAMTS-1 protein levels and the production of proteolytic cleaved versican in primary human granulosa cells.

To confirm the effects of BMP4 on ADAMTS-1 expression and the functions of versican cleavage in primary human granulosa cells, cells from three different samples were treated with BMP4 50 ng/ml for 24 hours. As shown in figure 5-3, BMP4 increased the protein levels of ADAMTS-1 and neoepitole of versican cleavage in all three tested samples.

5.3.4 SMAD4 is involved in generation of neoepitope of versican cleavage in human granulosa cells.

Given the fact that SMAD4 is essential in the BMP signaling pathway in the regulation of down-stream gene expression, we examined whether SMAD4 was involved in BMP4-induced cleavage of versican. SVOG cells were transfected with either vehicle or siSMAD4 (25 nM) for 48 hours and then treatment with BMP4 (30 ng/ml) for 24 hours. The mRNA levels of BMP4-

induced versican mRNA (Figure 5-4B) were blocked by knockdown of SMAD4. The protein levels of BMP4-induced ADAMTS-1 and the neoepitope generation from versican cleavage were attenuated by knockdown of SMAD4 (Fig. 5-4C). These results indicate that SMAD signaling is involved in the BMP4-induced ADAMTS-1 protein expression and the activity of versican cleavage.

5.4 Discussion

We describe, for the first time, the effects of recombinant BMP4 on ADAMTS-1 protein levels and the functions of versican cleavage in primary and immortalized human granulosa cells. At the time of ovulation, ADAMTS-1 was believed to play a critical role in the COC expansion and basement membrane breakdown [56, 73]. The substrate of ADAMTS-1, versican, was believed to localize and mediate the hyaluronan-rich COC matrix with the N-terminal hyaluronan-binding domain and the C-terminal lectin-like domain that binds to the cell surface [73]. During COC expansion, the volume of COC matrix increases 20 to 40 fold within hours with increasing in hyaluronan and hyaluronan-binding protein production [332]. ADAMTS-1 mediated versican cleavage may regulate the matrix reorganization and assist in the COC expansion. *Adamts-1* null female mice appear to have disrupted structural organization of the COC matrix with cumulus cells clumps and large cell aggregates because of impaired versican cleavage [323]. *Adamts-1* knockout mice also show increased anovulatory follicles with trapped mature oocytes [321], which indicated that ADAMTS-1 plays an important role in basement membrane breakdown and oocyte rupture.

ADAMTS-1 has been demonstrated to cleave the versican V0/V1 isoforms by the βGAG domain present in V0/V1 isoforms, generating two versican cleaved fragments: the C-terminal

G3 fragment with EGF-like domains and the N-terminal G1 domain with ECM binding affinity [56]. The proteolytic cleavage of versican by ADAMTS-1 is thought to augment the degradation of the basement membrane which is composed of full-length versican. However, the cleaved versican fragments have been found to exert their function by binding to cell surface or matrix molecules [333]. For example, the G1 domain of versican was reported to exert anti-apoptotic effects by down-regulating the pro-apoptotic molecule Bax [334, 335]. Interestingly, BMP4 was found to inhibit apoptosis in granulosa cells in some species, including cow and goat [235]. The G3 domain of versican has been found to promote angiogenesis by inducing VEGF expression and enhancing endothelial cell proliferation and migration [336]. Moreover, the EGF-like domains located on the C-terminal G3 fragments are able to then bind to EGF receptors on cell surfaces and promote cell proliferation [333].

In animal models, the expression of ADAMTS-1 was believed to be LH/hCG-induced and PR-regulated in ovaries [97, 337, 338]. Treatment of rats during the preovulatory period with the progesterone inhibitor, epostane, reduced ADAMTS-1 mRNA levels by 80%, and this inhibitory effect was overcome by exogenous progesterone [338]. Additionally, the up-regulation of ADAMTS-1 expression by hCG was abolished in *Pgr* knockout mouse ovaries [97]. Moreover, in rat granulosa cells, both PRA and PRB could activate the *Adamts-1* proximal promoter-luciferase reporter. The C/EBPB, nuclear factor 1-like factor, and three Sp1/Sp3 binding sites were included in the PRA activation regions on *Adamts-1* promoter [339]. The mature ADAMTS-1 was secreted by cleaving the prodomain with the putative furin proteolytic processing sites in human kidney cells [340]. Our recent findings that BMP4 increased furin expression as early as 3 hours in human granulosa cells provide a possible mechanism of the functions of BMP4 on ADAMTS-1 protein expression (starting at 6 hours) [341]. Moreover,

MMP2, MMP8, and MMP15 have been demonstrated to be able to cleave ADAMTS-1 at a spacer domain [342]. It cannot be ruled out that BMP4 increased ADAMTS-1 protein levels by up-regulating the production of these proteinases because BMPs (BMP2, BMP4, and BMP7) were found to stimulate EMT by up-regulating MMP2 in pancreatic cancer cells [343].

In summary, the present study demonstrates that BMP4 up-regulates ADAMTS-1 protein levels and functions of versican proteolytic cleavage in human granulosa cells. These findings provide important insights into the functional roles of BMP4 on COC expansion and follicle rupture in the periovulatory period.

5.5 Figures

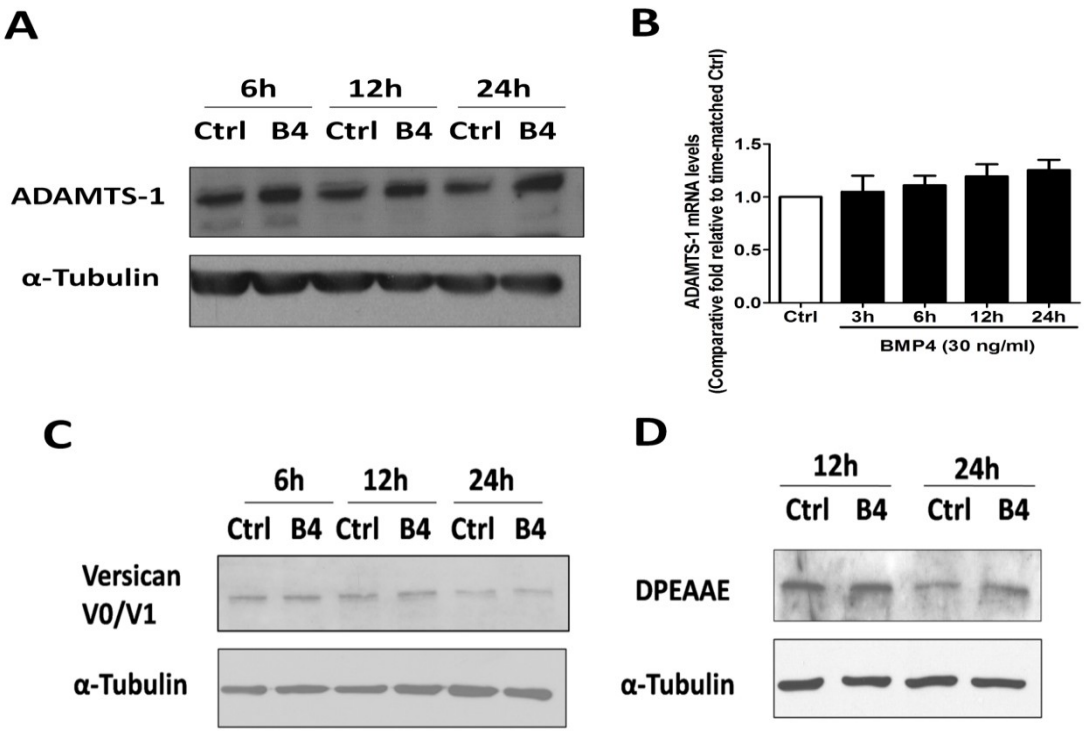

Figure 5-1 BMP4 up-regulates ADAMTS-1 protein levels and the production of proteolytic cleaved versican in SVOG cells.

SVOG cells were treated with 30 ng/ml of BMP4 for 3h, 6h, 12h and 24 h, and the mRNA levels of ADAMTS-1 (B) were examined by RT-qPCR. The protein levels of ADAMTS-1 (A), the full-length V0/V1 versican isoforms (C), and the neoepitope generated by cleavage of the versican core protein by ADMATS-1 (DPEAAE) (D) were examined by Western blot. GAPDH mRNA levels and α-Tubulin protein levels were used as internal controls. Results are expressed as the mean ± SEM of at least three independent experiments.

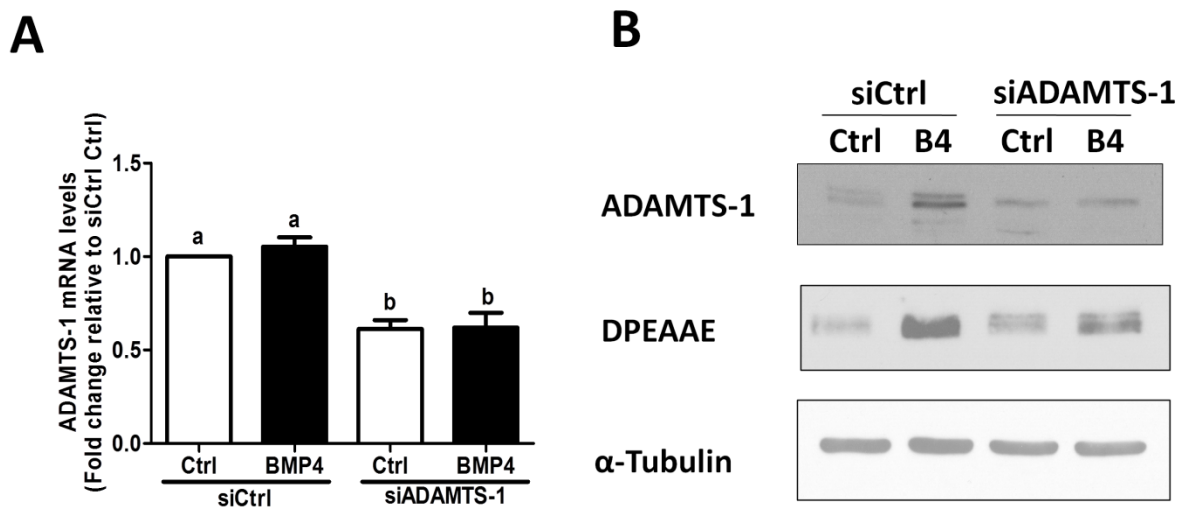

Figure 5-2 ADAMTS-1 is involved in BMP4-induced cleavage of versican in SVOG cells

SVOG cells were transfected with 10 nM control siRNA (siCtrl), or ADAMTS-1 siRNA (siADAMTS-1) for 48 hours, treated with BMP4 30 ng/ml for 24 hours. The knockdown efficiency of ADAMTS-1 was examined by RT-qPCR (A) and Western blot (B). The neoepitope of cleaved versican (DPEAAE) were examined by Western blot (B). GAPDH mRNA levels and α-Tubulin protein levels were used as internal controls. Results are expressed as the mean ± SEM of at least three independent experiments. Values marked by different letters are significantly different ($p<0.05$).

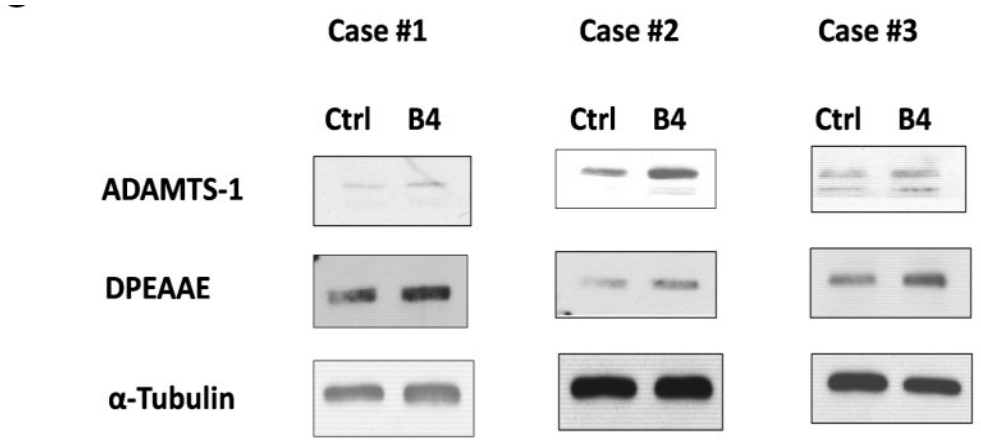

Figure 5-3 BMP4 up-regulates ADAMTS-1 protein levels and the production of proteolytic cleaved versican in primary human granulosa cells.

Primary human granulosa cells from three different samples were treated with 50 ng/ml of BMP4 for 24 hours. The protein levels of ADAMTS-1 and the neoepitope of cleaved versican (DPEAAE) were examined by Western blot. α-Tubulin protein levels were used as an internal control.

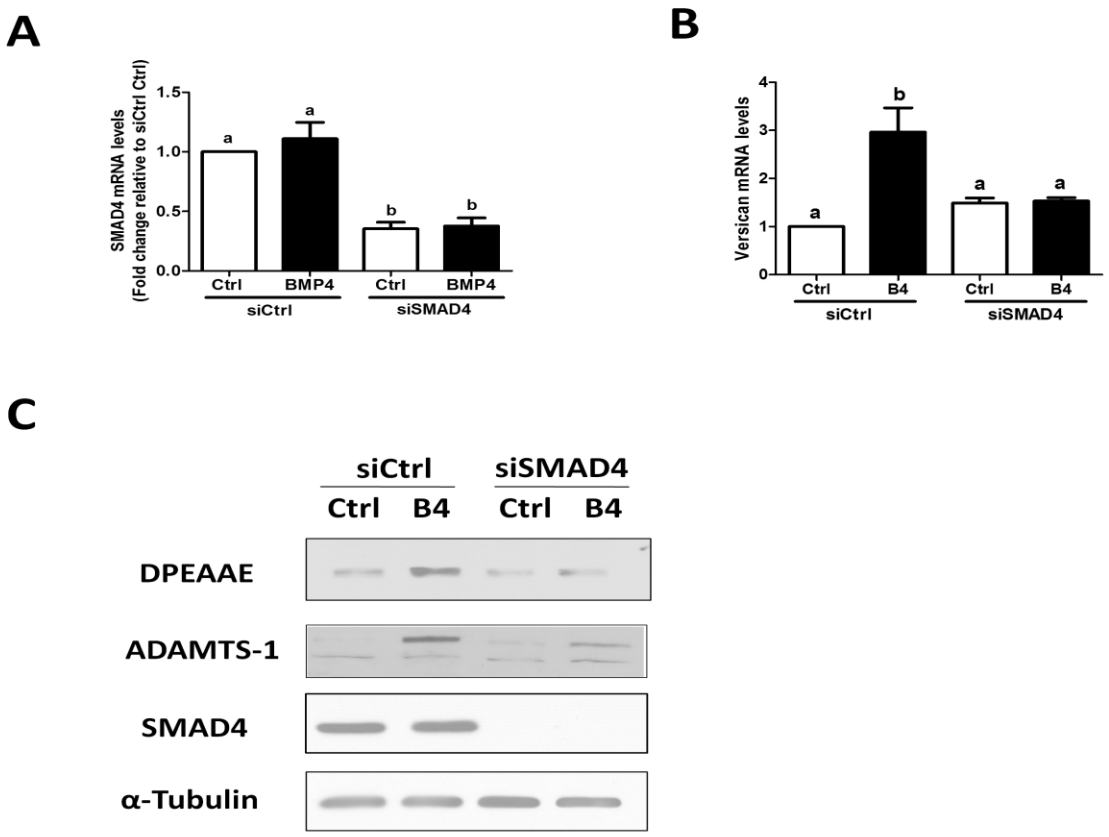

Figure 5-4 SMAD4 is involved in generation of the neoepitope of cleaved versican in human granulosa cells

SVOG were transfected with 25 nM control siRNA (siCtrl), or SMAD4 siRNA (siSMAD4) for 48 hours, treated with BMP4 30 ng/ml for 24 hours. The knockdown efficiency of SMAD4 was examined by RT-qPCR (A) and Western blot (C). The mRNA levels of versican (B) were examined by RT-qPCR. The protein levels of ADAMTS-1 and the neoepitope of versican cleaved by ADAMTS-1 were examined by Western blot (C). GAPDH mRNA levels and α-Tubulin protein levels were used as internal controls. Results are expressed as the mean ± SEM of at least three independent experiments. Values marked by different letters are significantly different ($p<0.05$).

Chapter 6: Conclusion, general discussion and future work

6.1 Conclusion

The main objective of my research work was to explore the functions of BMPs on human granulosa cells during the periovulatory interval. I examined the effects of recombinant BMPs on steroidogenesis, hyaluronan production, and the expression of ECM remolding gene (versican and ADAMTS-1) expression that play important roles in COC expansion and follicle rupture, and investigated the underlying mechanism in granulosa cells.

First, BMP4 and BMP7 suppress progesterone production and StAR expression while increase estradiol production and aromatase expression in human granulosa cells. Furthermore, our results suggest that the suppressive effects of BMP4 and BMP7 on StAR expression are mediated by ALK3 and canonical SMAD1/5/8 signaling.

Second, BMP4, BMP6, BMP7 and BMP15 all up-regulate HAS2 expression and hyaluronan production as well as the expression of the hyaluronan binding protein, versican, in human granulosa cells. Differential activation of noncanonical SMAD2/3 signaling by BMPs causes disproportionate induction of HAS2 expression and hyaluronan production. Moreover, the up-regulated effects of both noncanonical SMAD2/3 activation and HAS2 expression by BMP4 are reversed by SB-431542. This suggests that ALK4/5/7, as well as ALK2/3, is also involved in the biological functions of BMPs.

Third, the present study demonstrates that BMP4 stimulates ADAMTS-1 expression and the functions of versican cleavage in human granulosa cells. Moreover, the up-regulation of ADAMTS-1 activity by BMP4 is through SMAD-dependent signaling.

In summary, these results highlight several important roles of BMPs on morphological changes during the periovulatory interval. BMPs decreased progesterone/estradiol ratio, which

prevents premature luteinization, whereas increased hyaluronan production, versican expression, and ADAMTS-1 activity, which promotes COC mass formation and regulates both COC expansion as well as follicle rupture. Collectively, these data provide a new insight into the functions of BMPs during the ovulation/luteinization process. The BMPs may regulate the ovulation/luteinization process by promoting ovulation early events and preventing luteinization. In addition, the findings that ALK4/5/7 is involved in the effects that BMP4 has on HAS2 expression but not on StAR expression suggest human granulosa cells have the capacity to signal via multiple BMP type I receptors in response to these factors, but that the receptor(s) are differentially coupled to specific target genes or cellular responses. Furthermore, BMPs may exert different functions through different downstream SMAD signaling pathways: BMPs regulate progesterone production (maybe steroidogenesis) through canonical SMAD1/5/8 signaling, and regulate hyaluronan production (maybe COC expansion) through noncanonical SMAD2/3 signaling. Taken together, the findings in this thesis expanded the understanding of the physiological roles of BMPs during the periovulatory interval and of the ovulation process that is closely regulated and occurs with appropriate timing (Figure 6-1).

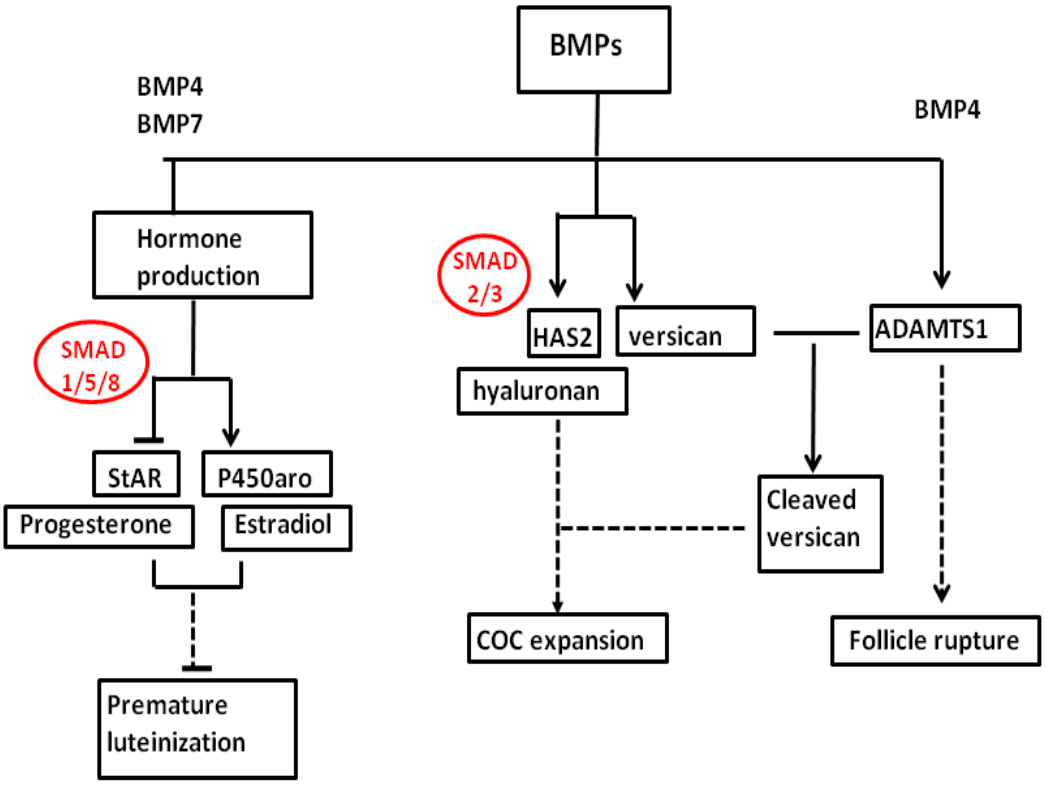

Figure 6-1 Summary of the roles of BMP signaling during the periovulatory interval

In human granulosa cells, BMP4 and BMP7 suppress progesterone production by down-regulating the expression of StAR but, they up-regulate both estradiol production and aromatase expression. BMPs could inhibit premature luteinization by decreasing the progesterone/estradiol ratio. BMP4, BMP6, BMP7 and BMP15 all up-regulate HAS2 expression and hyaluronan production as well as the expression of the hyaluronan-binding protein versican. BMPs could stimulate COC mass formation by stimulating hyaluronan production and regulate COC expansion by increasing versican proteolytic cleavage by ADAMTS-1. BMP4 stimulates ADAMTS-1 protein expression that could contribute to follicle rupture. BMPs regulate progesterone production through canonical SMAD1/5/8 signaling, and regulate hyaluronan production through noncanonical SMAD2/3 signaling.

6.2 General discussion

6.2.1 Human granulosa cells during the periovulatory interval

Granulosa cells are somatic cells surrounding ooctyes, and are themselves surrounded by theca cells, with a basement membrane between those two types of cells. The functions of granulosa cells include steroid hormone production and growth factors secretion, which are essential for oocyte maturation and ovulation. In human beings, granulosa cells, from IVF patients with oocyte retrieval, are the most valuable and practicable tool for studies of ovarian functions. The granulosa cells express most of the genes involved in steroidogenesis (including StAR and aromatase), follicle development (including AMH), COC expansion (including HAS2 and PTX3), oocyte rupture (including COX2 and ADAMTS-1) and angiogenesis (including VEGF). These granulosa cells are widely used for ovarian function study, including angiogenesis [344], ovarian reserve [345], as well as all hormone production [247, 282, 346, 347]. In our lab, an immortalized human granulosa cell line, SVOG cells, that preserves most of the characters of primary granulosa cells has been used for studies on ovulation [107, 108, 263, 303, 348], cumulus cell expansion [283, 349], hormone production [117], and apoptosis [350]. In my studies, I used both primary granulosa cells and SVOG cells to investigate the functions of BMP signaling during the periovulatory interval. I am aware that both primary and SVOG granulosa cells undergo luteinization in culture, and are not a perfect cell model for studying follicular-luteal transition. However, since we are not able to culture human preovulatory follicles or COCs, these granulosa cells provide available cell models for studying the genes expression changes regulated by ovulation regulators in the morphological changes during the periovulatory interval [107]. Both primary granulosa cells and SVOG cells used in my study are obtained from IVF patients, and are more like mural granulosa cells with more steroidogenesis [351] and

angiogenesis potential [352]. However, mural granulosa cells obtained from mouse antral follicles were similar to cumulus cells to synthesize and accumulate hyaluronan *in vitro* in response to the oocyte and gonadotropins. It suggested that the mural granulosa cell cultures could be used to facilitate the studies of the OSFs functions on regulation of the COC expansion-related gene expression *in vitro* [112].

6.2.2 BMPs regulate steroid hormone production in species-specific and stage-specific manners during folliculogenesis.

The effects of BMPs on ovarian steroidogenesis appear species-specific differences in preantral and antral follicles. In rat granulosa cells of preantral follicles, Otsuka *et al.* indicated that oocyte-derived BMP15 had a marked effect on down-regulation of FSH-induced progesterone production but had no effect on FSH-induced estradiol production [300]. They further indicated that BMP15 regulated FSH-induced steroid hormone production and StAR, P450scc, 3β-HSD, P450arom, LHR, and inhibin subunits by down-regulating FSH receptor expression but not at the basal levels [353]. Moreover, they also found BMP6, another oocyte-derived BMP, had a similar effect on FSH-induced steroid hormone production and enzyme expression. However, BMP6 exerted its effects by decreasing adenylate cyclase activity [298]. Theca cell-derived BMP4 and BMP7 enhanced FSH-induced estradiol and decreased progesterone production, but had no effect on basal levels [260]. In summary, in rat preantral follicles, BMPs can regulate gonadotropin-induced steroid hormone production and enzyme expression, but do not affect the basal levels.

Briefly, in ruminant antral follicles, BMPs regulate both basal levels and gonadotropin-induced hormone production. In bovine antral follicles, BMP4, BMP6 and BMP7 enhanced basal

and IGF-stimulated estradiol but suppressed progesterone production in granulosa cells [248]. BMP4, BMP6 and BMP7 were also found to reduce basal and LH-induced androgen secretion, and the expression of P450c17, P450scc, 3β-HSD and StAR in theca internal cells [278]. In sheep granulosa cells of small antral follicles, BMP4 decreased both basal and FSH-stimulated progesterone secretion as well as StAR and P450scc expression [261]. BMP2, BMP4, BMP6 and BMP7 were found to inhibit basal progesterone production without affecting cell proliferation [234].

A recent study demonstrated stage-specific effects of BMP on steroid hormone secretion by invading recombinant human BMP6 infusion into sheep ovarian artery [227]. During the early follicular phase, BMP6 injection induced an acute stimulation of inhibin A, androstenedione and estradiol, which resulted in a precocious LH surge and ovulatory follicle maturation, and a smaller corpora lutea in smaller size follicles. At the luteal regression phase, BMP6 injection had no effect on LH-induced estradiol, androstenedione or inhibin A. The ovulation rate had no difference between the control and BMP6 treatment groups [227].

In humans, some studies have shown inconsistent results on regulation of ovarian steroid hormone secretion by BMPs. In KGN granulosa tumor cells, BMP4 and BMP7 decreased forskolin and dibutyryl-cAMP-induced progesterone production, but did not affect either forskolin- or BtcAMP-induced estradiol production [40]. Using granulosa cells from IVF patients, it was shown that BMP2 decreased StAR but increased aromatase expression [247]. By contrast, in another prospective cohort study performed on granulosa cells between normal cycle women and PCOS patients, BMP7 but not BMP4, decreased basal estradiol production while neither BMP4 nor BMP7 had effect on FSH-induced estradiol production in healthy women

[246]. Our previous study found that BMP15 decreased progesterone production and StAR expression in SVOG immortalized granulosa cells but not in KGN granulosa tumor cells [118].

6.2.3 BMPs could prevent premature luteinization by decreasing the progesterone estradiol ratio

In my study, both BMP4 and BMP7 decrease the progesterone estradiol ratio by suppressing progesterone production while increasing estradiol production in human granulosa cells. These results indicated that BMP4 and BMP7 may prevent premature luteinization, which is defined as a progesterone estradiol ratio > 1 during the periovulatory interval in IVF patients undergoing controlled ovarian hyperstimulation (COH) [354]. Premature luteinization may also adversely affect the pregnancy rate [355] due to impaired endometrial receptivity [356, 357] and reduced implantation [358], with [359, 360] or without [357, 361] impaired oocyte-embryo quality.

The mechanism of negative impact on endometrial receptivity by an early progesterone rise is unclear. However, advanced endometrial histologic features and an absence of pinopods, a functional marker of uterine receptivity, were observed at the time of embryo implantation in high progesterone group samples [362]. They speculated that the early rise progesterone might cause a premature appearance of endometrial implantation window, which might result in the decrease of endometrial receptivity. Another study suggested that using low doses of antiprogesterone could correct the early endometrial implantation window caused by early progesterone rise in COH patients [363].

6.2.4 BMPs could promote COC mass production and regulate COC expansion

The functions of BMP signaling on COC expansion have been investigated in animal models. BMP15 and BMP6 have been detected in the oocytes of rats and sheep [202, 203, 234], and shown to play important roles in COC expansion both *in vivo* and *in vitro*. For example, mice deficient of BMP15 or BMP6 display reduced ovulation rates accompanied by impaired COC expansion [201, 229]. *In vivo*, recombinant human BMP15 has been shown to stimulate COC expansion and COC expansion-related gene expression in mice [110], cows [205] and pigs [206]. In mouse ovaries, recombinant human BMP15 stimulated COC expansion by up-regulating expression of EGF-like ligand (amphiregulin, betacellulin and epiregulin) and through EGF signaling [110]. BMP15 also induced COC expansion in bovine ovaries by up-regulating hyaluronan production and glucose uptake [205]. In porcine COC *in vitro* maturation, BMP15 induced the expression of genes involved in oocyte maturation (e.g. cdc2), and COC expansion (e.g. HAS2) [206]. By contrast, the addition of BMP2 or BMP4 to *in vitro* maturation medium did not affect COC expansion in cows [241].

Our previous studies in human granulosa cells have yet to identify difference in the functions of BMP4, BMP7 and BMP15, at least with respect to the regulation of StAR and connexin 43. My study (CHAPTER 4) confirmed the functions of BMPs on COC expansion in animals, and I found BMP4, BMP6, BMP7 and BMP15 all induced HAS2 expression and hyaluronan production, suggesting that these BMPs could participate in COC formation in human granulosa cells. My results suggest that BMPs also increase the expression of the hyaluronan binding protein versican, which has been demonstrated in the COC matrix though its functions have yet to be fully defined. Interestingly, BMPs have been shown to regulate ECM expansion by stimulating hyaluronan production and versican expression during endocardial

cushion [149, 182, 364] and renal corticointerstitium [365] development. Thus, BMP signaling may have a general biological function to regulate ECM expansion and EMT by stimulating HAS2, hyaluronan and hyaluronan-binding versican in different systems.

Our previous studies demonstrated that BMP4 and BMP7 inhibited PTX3 accumulation in human granulosa cells [283]. In mouse models, deficient of light chain of IαI (no intact IαI), TSG-6, or PTX3, results in a dysfunctional matrix and female infertility [293, 294, 366, 367]. However, the concentrations of these regulator molecules, and how these molecules participate in COC expansion remains unknown, especially since PTX3 does not appear to bind hyaluronan directly. In the complicated COC matrix, there are two types of fundamental tertiary hyaluronan-binding complex: hyaluronan-IαI HC-IαI HC and hyaluronan-TSG-6-IαI HC (Figure 1-1). The presence of IαI decreased the binding and cross-linking between TSG-6 and hyaluronan [53]. A recent study found that PTX3 cannot interact with TSG-6 alone, nor incorporate into hyaluronan films that have been previously exposed to IαI and TSG-6. PTX3 can bind to TSG-6 of the hyaluronan-TSG-6-IαI HC tertiary hyaluronan-binding complex. This will only occur if PTX3 previously encounter with IαI. The hyaluronan films with PTX3 incorporation became more condensed [54]. This study is consistent with the *Ptx3* knockout mice study in that the covalent modification of hyaluronan with IαI HCs were not disturbed because of deficiency of PTX3 [367]. Thus, PTX3 is as a regulator of the matrix, and mostly on stability and condensation. Additionally, BMP4 also stimulated ADAMTS-1 activity (CHAPTER 5), and cleavage of COC matrix protein versican. Moreover, *Adamts-1* null female mice appear disrupted structural organization of the COC matrix with cumulus cells clumps and large cell aggregates because of impaired versican cleavage [323]. Based on these studies in our lab, BMPs might promote COC formation by stimulating hyaluronan synthesis and hyaluronan-binding protein expression, while

simultaneously loosing the matrix by decreasing PTX3 and increasing ADAMTS-1 activity of versican cleavage (Figure 6-2).

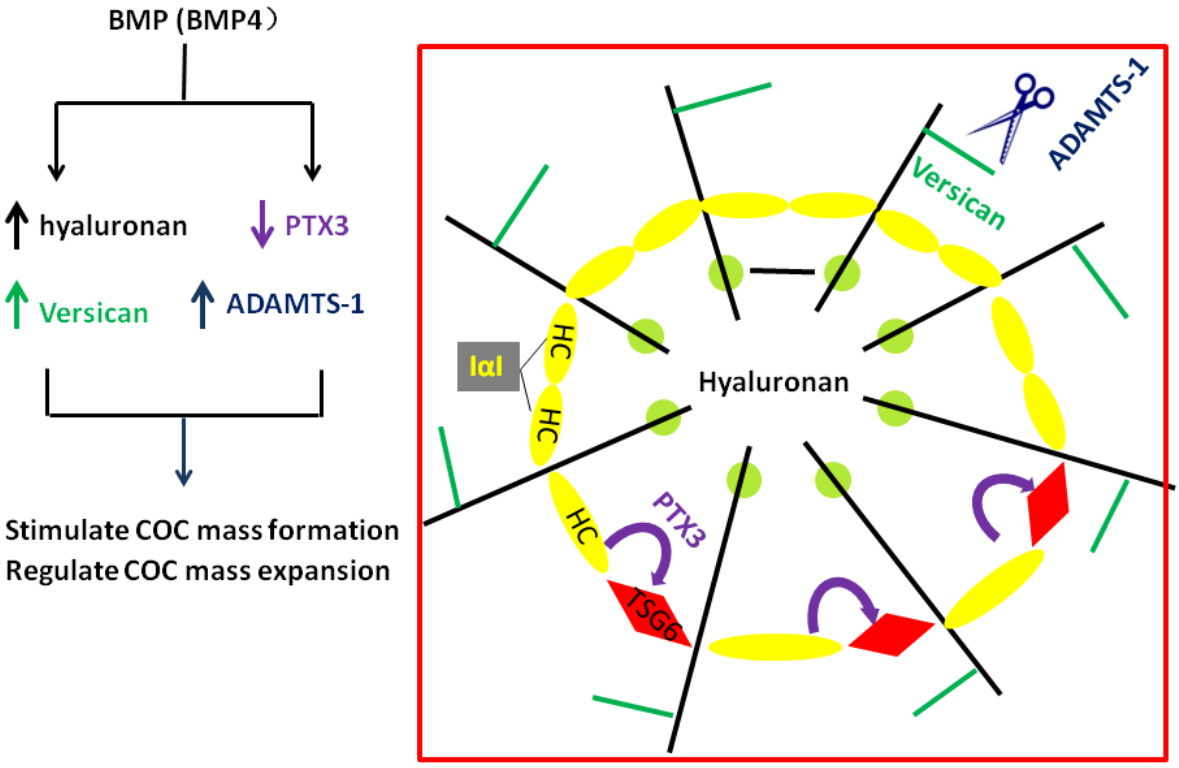

Figure 6-2 BMP4 may promote COC mass formation and regulate COC expansion

There are two types of fundamental tertiary hyaluronan-binding complex: hyaluronan-IαI HC-IαI HC and hyaluronan-TSG-6-IαI HC. PTX3 can bind to the TSG-6 of the hyaluronan-TSG-6-IαI HC tertiary hyaluronan-binding complex. Versican, a proteoglycan, binds to hyaluronan directly and is proteolytically cleaved by ADAMTS-1. BMP4 may promote COC formation by stimulating hyaluronan synthesis and versican expression, while simultaneously loosing the matrix by decreasing PTX3 and increasing versican proteolytic cleavage by ADAMTS-1.

6.2.5 BMPs may contribute to follicle rupture and cell-cell gap-junction reduction

Based on the knockout mice studies, ADAMTS-1 is believed to have a nonredundant role in follicle rupture [321, 323]. Nevertheless, ADAMTS-4 has a similar function of versican cleavage and is also abundantly expressed in periovulatory follicles [56]. *Adamts4* null mice were fertile with no ovarian defects, and *Adamts4*/*Adamts1* double mutant granulosa-specific null ovaries showed the identical phenotype as *Adamts1* null mice [323]. In my study, the findings that BMP4 induced both ADAMTS-1 protein expression and the functions of versican cleavage suggested that BMP4 could stimulate follicle rupture.

Additionally, our previous studies have shown that BMPs (BMP4, BMP7 and BMP15) down-regulated connexin 43 in human granulosa cells [263, 303]. Connexin 43 is down-regulated by LH in granulosa cells [58] and by PR in various human tissues, especially in myometrial cells [368-372]. In porcine granulosa cells, the decreased connexin 43 mRNA by FSH+LH cultivation as reversed by treatment with RU 486 [373]. In rats, follicles incubated with LH or injected with hCG exhibited down-regulated connexin 43 expression and a subsequent loss of intercellular coupling within the granulosa cell layers [374-377]. Moreover, this reduction of cell-cell communication results in decreases cGMP signaling and oocyte meiosis resumption [57, 58, 60]. Thus, BMPs might stimulate oocyte meiosis resumption and ovulation by down-regulation of connexin 43.

6.2.6 BMPs exert different functions by differential activation of canonical SMAD1/5/8 and noncanonical SMAD2/3 signaling

Previous work in our lab examined the signaling pathways involved in a variety of BMP-regulated functions in granulosa cells, including the specific TGF-β type I receptors activated by

different BMPs, and the effects of TGF-β type I inhibitors on BMP functions [118, 283, 303, 341]. However, my results suggest the signaling of BMPs more complicated than previously thought.

First, different BMPs have similar functions and regulation of gene expression, but the strength of effects varies, depending on the activation of different signaling. In SVOG cells, both BMP4 and BMP7 decrease StAR (CHAPTER 3), connexin 43 [303] and PTX3 [283], and increase HAS2 (CHAPTER 4), versican (CHAPTER 4), inhibin βA, furin [341] and COX2 (Appendix B) expression. Compared to BMP7, BMP4 had a stronger effect on StAR, connexin 43 expression regulation. However, equal effects has been shown on regulation of PTX3 [283], HAS2 (CHAPTER 4), versican (CHAPTER 4), inhibin βA [341], furin [341] and COX2 (Appendix B), even though in all those studies BMP4 had a stronger activation of canonical SMAD1/5/8. My results suggest that equivalent effects of BMP4 and BMP7 may arise from noncanonical SMAD2/3 signaling, as is the case for HAS2, and this could help explain other studies where biological functions do not entirely correlate with the degree of canonical SMAD1/5/8 activation.

Second, BMPs regulate biological process in granulosa cells by activating both canonical SMAD1/5/8 and noncanonical SMAD2/3 signaling. In studies of BMPs on StAR expression (CHAPTER 3), the strength of effects on StAR expression regulation by BMP4 and BMP7 is consistent with the strength of SMAD1/5/8 activation. Consistently to my results that BMP4 induced a stronger activation of SMAD1/5/8 than BMP15 (CHAPTER 4), BMP15 was required at a higher concentration to down regulate StAR, compared to BMP4 [118]. Moreover, the down-regulation of StAR expression by BMP4 cannot be affected by SB-431542 (ALK4/5/7 inhibitor), which does not inhibit BMP4-induced SMAD1/5/8 activation. These results are

consistent with the effects of inhibitors on SMAD1/5/8 activation, but not on SMAD2/3 activation (SB-431542 totally inhibited SMAD2 activation and partially inhibited SMAD3 activation). Additionally, knockdown both SMAD2 and SMAD3 did not alter the effects of BMPs (BMP4 and BMP7) on StAR expression (CHAPTER 3), but blocked the effects of BMPs (BMP4 and BMP15) on HAS2 expression (CHAPTER4). These results suggested that BMPs might regulate progesterone production (probably steroidogenesis) through canonical SMAD1/5/8, whereas regulate hyaluronan production (probably COC expansion) through noncanonical SMAD2/3 signaling (Figure 6-3).

Third, interestingly, human granulosa cells have the capacity to signal via multiple TGF-β type I receptors (both ALK2/3/6 and ALK4/5/7) in response to BMP ligands, but that different receptor(s) may somehow be coupled to different target genes and/or functions. In my study, BMP4-induced activation of canonical SMAD1/5/8 signaling was totally blocked by dorsomorphin (inhibitor of ALK2/3/6), mostly reversed by DMH1 (inhibitor of ALK2/3), but not affected by SB-431542 (ALK4/5/7). The activation of noncanonical SMAD2/3 signaling was totally blocked by dorsomorphin, mostly reversed by DMH1 and SB-431542 (CHAPTER 4). This opens up many possibilities since most ALKs, even from different groups, can form heterodimers [140]. Nevertheless, most of the effects of BMPs on downstream gene regulation in our studies cannot be blocked by SB-431542. The BMP4-induced up-regulation of HAS2 (CHAPTER 4) and furin [341] were partially reversed by SB-431542, indicating ALK4/5/7 were involved in the effects of BMP4 on these two genes expression regulation. Additionally, our previous studies found that differential subsets of BMP type I receptors mediate the effects of BMP4 (ALK3/6) and BMP7 (ALK3/2) on pentraxin 3 [283]. By contrast, in my study, the new finding that the effects of BMP4 and BMP7 on StAR expression are mediated exclusively by

ALK3 suggests that the BMP type I receptor(s), activated by a certain BMP, might be differentially coupled to specific target genes. Differential involvement of ALKs could explain the differences in phenotypes between *Alk* knockout mice, which were suggested to be indication of nonredundant functions of ALK3 and ALK6 on ovarian functions. The *Alk3* knockout female mice were subfertile with significantly reduced ovulation rates, but normal COC expansion. The *Alk 6* knockout mice exhibited increased multi-oocyte follicles [253], and defects of COC expansion with decreased COX2 expression [254]. In summary, ALK3 is involved in spontaneous ovulation and ALK6 is essential for COC expansion [253, 254, 378].

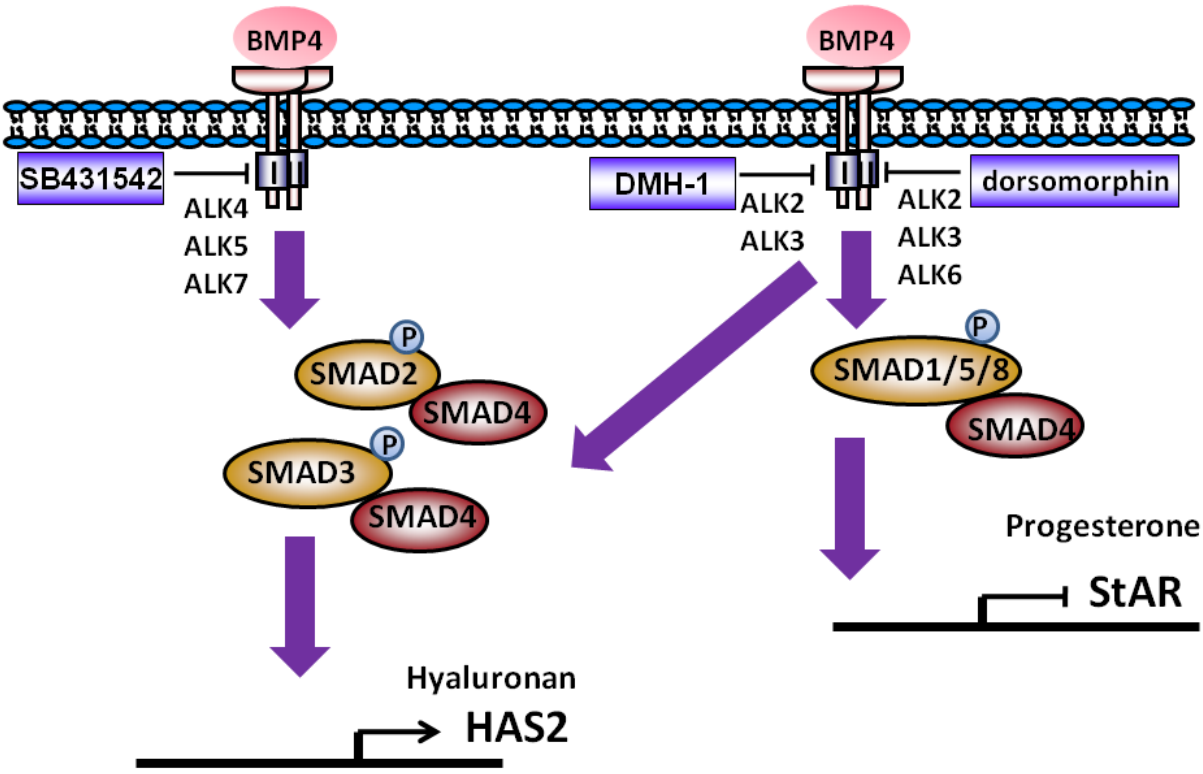

Figure 6-3 BMP4 exerts different functions through different SMAD signaling

BMP4 activates both canonical SMAD1/5/8 signaling and noncanonical SMAD2/3 signaling. BMP4-induced activation of canonical SMAD1/5/8 signaling is totally blocked by dorsomorphin (inhibitor of ALK2/3/6), mostly reversed by DMH1 (inhibitor of ALK2/3), but not affected by SB-431542 (ALK4/5/7). The activation of noncanonical SMAD2/3 signaling is totally blocked by dorsomorphin, mostly reversed by DMH1 and SB-431542. These results indicate that ALK2/3 is essential for BMP4-induced both canonical SMAD1/5/8 and noncanonical SMAD2/3 activation, while ALK4/5/7 is only involved in noncanonical SMAD2/3 activation. In addition, BMP4 exerts different biological roles by activating different SMAD signaling: down-regulating progesterone production through canonical SMAD1/5/8 signaling while up-regulating hyaluronan production (probably COC expansion) through noncanonical SMAD2/3 signaling.

6.2.7 Limitations

SVOG cells and primary human granulosa cells are useful *in vitro* cell models for investigating the functions and mechanisms of BMPs during the periovulatory interval. Nevertheless, they possess some limitations where are important to consider. First, without oocytes co-culture, these granulosa cells are more similar as luteinized granulosa cells, and are not perfect to investigate the other phases of folliculogenesis. Second, the functions of BMPs on steroid hormone production have not been confirmed by serum hormone measurement in human. As we known, the main functions of both estradiol and progesterone are dependent on serum concentration: estradiol affects the feedback loop to regulate folliculogenesis and menstrual cycle, while progesterone exerts its main function in uterus for pregnancy. Second, the effects of BMPs, as well as other oocyte secreted factors (e.g. GDF9), on COC expansion have not been confirmed *in vivo* in humans. Moreover, the functions of versican and ADAMTS-1 in COC expansion have not been investigated in animal models. In a summary, to confirm physiological relevance of BMPs during periovulatory interval, more clinical retrospective evaluation studies are necessary.

6.3 Clinical potential

These studies also provide a new sight for future clinical roles of BMPs in the ovulation and luteinization processes. The new knowledge might help to improve IVF protocols, and investigate infertility-related diseases, such as PCOS, obesity, and endometriosis. **First, BMPs might be used as biological markers for evaluation of ovarian functions.** BMP15/GDF9 mRNA levels were shown to be closely correlated to the human oocyte maturation, fertilization and embryo quality [120]. **Second, BMPs might be applied on *in vitro* maturation system:**

BMP15 and GDF9 were used to induce oocyte maturation and COC expansion in porcine IVM culture [206]. My findings indicate that, like BMP15/GDF9 heterodimers, BMP4 and BMP7 can both induce SMAD2/3 and SMAD1/5/8 signaling, and therefore could be of use in human IVM in the future. **The third, BMPs could be used to prevent the early endometrial implantation window in IVF patients undergoing COH.** Until now, BMP7 and BMP2 have been widely used in clinical treatments on spinal fusion and fracture healing [239]. Based on my study, BMPs (especially BMP4) have potential to decrease the progesterone estradiol ratio, but not adversely affect COC expansion and oocyte rupture. These results lead to a possibility to use BMP as an alternative treatment, instead of antiprogesterone, to correct the negative effects of early progesterone rise in COH patients [363] (Figure 6-4).

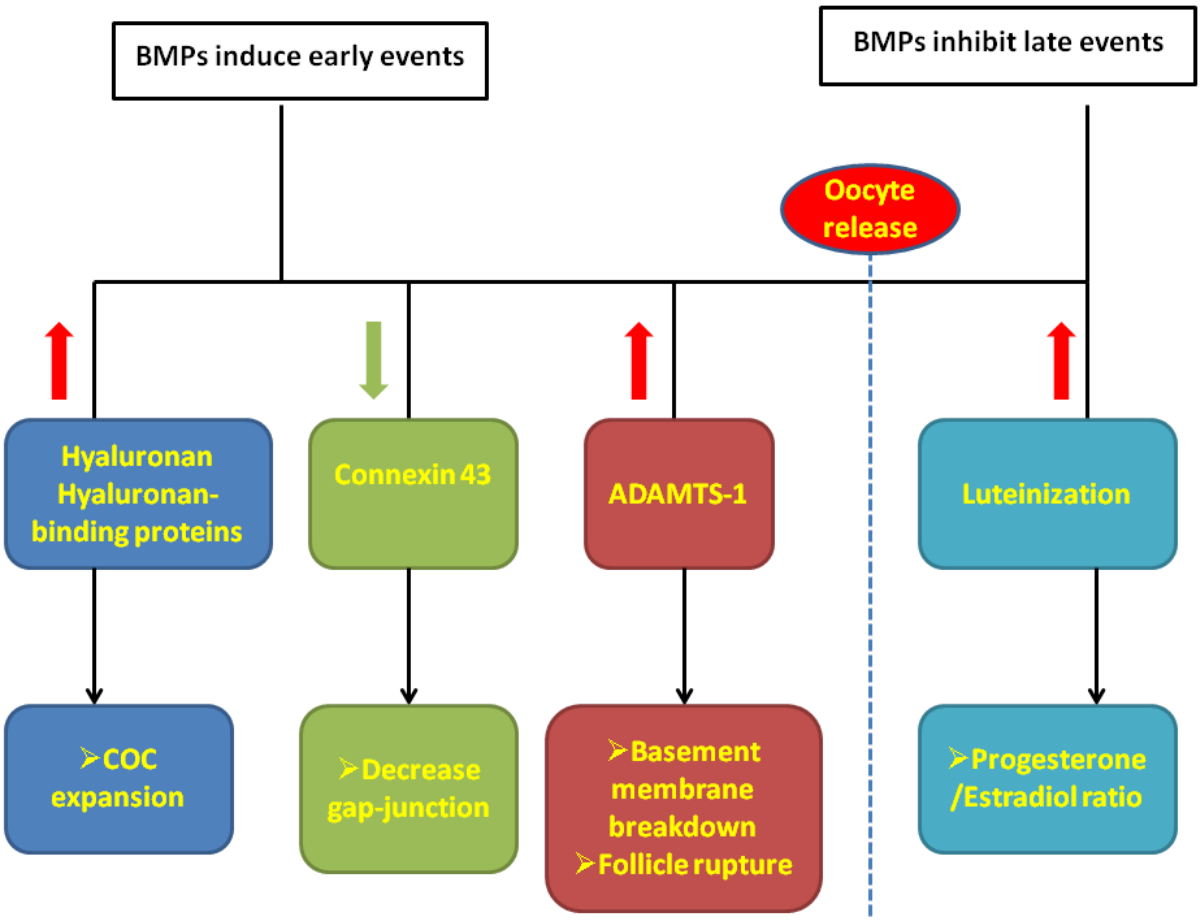

Figure 6-4 Clinical potential of BMPs on ovulation and luteinization

During the periovulatory interval, BMPs could promote COC formation by stimulating hyaluronan production and assist COC expansion by regulating hyaluronan-binding proteins production. BMPs decrease cell-cell gap-junction by decreasing connexin 43 expressions. BMPs could stimulate follicle rupture by up-regulating ADAMTS-1 activity. At the same time, BMPs prevent early luteinization by decreasing the progesterone estradiol ratio. These findings indicate BMPs regulate the appropriate timing of ovulation/luteinization process by promoting early events (COC expansion) and inhibiting late events (luteinization).

6.4 Future work

These results advance our understanding of the regulatory roles and the signaling pathway of BMPs in human granulosa cells. However, the correlations of BMPs and other molecular regulators during the periovulatory interval are still far from being fully understood. Further studies are needed to improve our knowledge with regards to the development of human granulosa cells.

6.4.1 The research on molecular regulations of BMPs expression

The spatiotemporal expression pattern of BMPs and their receptors in the ovary have been investigated in several species, including rats and cows [202, 240]. However, the expression pattern of BMPs in human ovaries is not well understood. Moreover, research into the regulation of the expression BMPs in the ovary is limited in any species. According to my preliminary data, there are high mRNA levels of BMP8A, BMP8B and BMP6, low levels of BMP2 mRNA, and no BMP5 mRNA in granulosa cells (Appendix A). Research on both BMP8A and BMP8B is limited. In my study, both BMP8A and BMP8B had no effect on expression of the BMP target genes StAR and ID-1 and also no effect on SMAD signaling activation (Appendix A). For future work, it will be important to investigate whether BMP4, BMP7, and BMP15 are expressed in human granulosa cells, and whether gonadotropins (LH, FSH) or other growth factors (EGF-like ligands) regulate BMPs expression.

6.4.2 The studies on different BMPs and signaling pathways

Until now, most studies on the ovarian functions of BMPs have shown that they exert similar effects on steroidogenesis. However, some studies have shown different, and in some

cases opposing expression patterns of these BMPs during folliculogenesis. Nevertheless, it suggests that different BMPs might exert different biological roles. Moreover, there is evidence to suggest that different BMPs exert similar biological roles through different signaling in granulosa cells. For example, BMP4 and BMP7 were found to inhibit granulosa cells apoptosis through different SMAD-independent pathways: BMP4 via PI3K/PDK-1/Akt and BMP-7 via PI3K/PDK-1/PKC [301]. Similarly, BMP15 and BMP6 were found to inhibit FSH-induced hormone production by different mechanisms: BMP15 down-regulating FSH receptors [353] and BMP6 decreasing adenylate cyclase activity [298].

In my studies, it was demonstrated that differential activation of noncanonical SMAD2/3 by BMP4, BMP6, BMP7, and BMP15 caused disproportionate induction of HAS2 expression. BMP15 was demonstrated to play an important role in ovulation both *in vivo* and *in vitro*. My studies suggest that BMP15 exerts mild effects on HAS2 and hyaluronan production (CHAPTER 4), whereas it may have more prominent effects on COX2 expression (Appendix B). Future, studies could investigate whether SMAD-independent signaling is involved in BMP15-induced COX2 expression, the regulation of other genes involved in folliculogenesis, and oocyte maturation and ovulation.

6.4.3 Research into the crosstalk between BMPs and steroid hormone receptors during the periovulatory interval

During the periovulatory interval, both progesterone and estradiol dramatically increase. However, the functions and mechanisms of either progesterone or estradiol during ovulation and luteinization are not well defined. In knockout mouse models of steroid hormone receptors, ER-β displayed reduced COC mass and ovulation rate [100]. By contrast, PR knockouts do not appear

to have any defects in COC expansion or luteinization but do display deficiencies in follicle rupture [95-97]. Additionally, BMP15 and GDF9 have been shown to be essential to coordinate with estradiol to promote COC expansion and HAS2 expression in mice [77]. Future studies could investigate the crosstalk roles between BMPs and PR or ER on gene expression regulation related to ovulation and luteinization.

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Appendices

Appendix A Granulosa cell-derived Bone Morphogenetic Protein 8A has no effect on ID-1 and StAR expression

A.1 Introduction

The expression patterns of BMPs in human ovarian cells have not been fully established. A study using immunohistochemistry and *in situ* hybridization shows that the proteins of BMP4 and BMP7 were detected in oocytes, granulosa cells and theca cells, while the mRNAs were detected only in theca cells in women ovaries [243]. Another study shows that the mRNAs of both BMP4 and BMP6 were detected in human corpora lutea including both granulosa-lutein cells and theca-lutein cells [244]. Both mRNA and protein expression of BMP15 were detected in oocytes, granulosa cells and stoma cells in girls and adults [245]. A study of the BMP expression in human granulosa cells of both healthy and PCOS women shows that the mRNAs of BMP2, BMP4, BMP5, BMP6, BMP7 and BMP8A were detected by RT-qPCR. Moreover, BMP6 has the predominant expression in healthy women and overexpression in PCOS women [246]. Another study demonstrated that the mRNA of BMP2 was detected in granulosa cells of antral follicles but not in the corpus luteum in IVF patients [247].

BMP8 plays a critical role in spermatogenesis and epididymis development in the male reproductive system [157, 197, 198]. However, there are no studies on BMP8 in the female reproductive system. Our preliminary results showed that relative high mRNA levels of BMP8A, BMP8B and BMP6, compared to BMP2 and BMP5, were detected in both primary and immortalized SVOG human granulosa cells. However, recombinant human BMP8A affected neither the expression of either StAR or inhibitor of DNA binding factor (ID-1), nor the activation of either canonical SMAD1/5/8 or noncanonical SMAD2/3 signaling.

A.2 Materials and Methods

Culture of SVOG immortalized human granulosa cells

SVOG is an SV40 large T antigen immortalized nontumorigenic human granulosa cell line [265]. Cells were grown in phenol-red free DMEM/F12 medium (Invitrogen) containing 10% charcoal-treated fetal bovine serum (FBS; Hyclone Laboratories Inc., Logan, UT). Cells were maintained at 37°C atmosphere with 5% CO_2, changing medium every other day. Cells were passaged by using trypsin-EDTA solution (0.05% trypsin, 0.5mM EDTA, Invitrogen), when they reached 90% confluence.

Primary human granulosa cells culture

Human granulosa cells were obtained from 2 IVF patients with agreement as described previously [379]. Follicular fluid from one individual patient was equally divided into 50 ml centrifuge tubes and centrifuges 400 × g for 20 min. After removing the supernatant, the cell pellet was resuspended in 4 ml DMEM/F12 medium (Invitrogen) containing 10% charcoal-treated fetal bovine serum (FBS; Hyclone Laboratories Inc., Logan, UT), 1X Antibiotic-Antimycotic (Anti-Anti; Life technologies, Grand Island, NY) and 1X Gluta MAX (Life technologies, Grand Island, NY). The cell suspensions were layered on 8 ml Ficoll-Paque Plus (Amersham Bioscience, Piscataway, NJ) in 15-ml sterile tubes and centrifuged at 4000 × rpm for 20 min. The cell layer was removed from Ficoll-Paque column and washed with 5 ml Medium and resuspended in 5 ml medium. Cells were counted and seeded 0.5-2 × 10^5 per well in 24-well culture plates and cultured for 48 hours before treatments.

Antibodies and reagents

Polyclonal rabbit anti-StAR, anti-SMAD1/5/8, monoclonal mouse anti-α-Tubulin were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Polyclonal rabbit anti-phosphor-SMAD1/5/8 ($Ser^{463/465}$, $Ser^{463/465}$, $Ser^{426/428}$, respectively), monoclonal rabbit phospho-SMAD2 ($Ser^{465/467}$), monoclonal mouse SMAD2, monoclonal rabbit phospho-SMAD3 ($Ser^{423/425}$), monoclonal rabbit antibody SMAD3, anti-Smad4 were obtained from Cell Signaling Technology (Beverly, MA). Recombinant human BMPBA was obtained from R&D Systems (Minneapolis, MN).

RT-qPCR

Total RNA was extracted with TRIzol Reagent (Invitrogen), and 2 μg of that was performed reverse transfection by using random primers and MMLV reverse transcriptase (Promega, Madison, WI). The mRNA levels of BMP2, BMP5, BMP6, BMP8A and BMP8B were examined by TaqMan gene expression assays (Applied Biosystems, Burlington, ON). Real-Time PCR was performed using the Applied Biosystems 7300 Real-Time PCR System equipped with 96-well reaction plate. The specificity of each assay was evaluated by dissociation curve analysis and agarose gel electrophoresis of PCR product. The mRNA levels were determined by comparative Ct ($2^{-\Delta\Delta Ct}$) method with GAPDH as the reference gene.

Western blot analysis

Cells were washed twice by cold PBC and then lysed with cell lysis buffer (Cell signaling), which contains protease inhibitor cocktail (Sigma-Aldrich). Equal amounts of protein (50 μg)

were separated by 10% SDS polyacrylamide gel electrophoresis and transferred to PVDF membrane. After blocking for 1 h with 5% nonfat dried milk in TBS containing 0.1% Tween 20, the membranes were incubated overnight at 4 °C with primary antibodies, which were 1:000 diluted in 5% nonfat dried milk. After washing, the membranes were incubated with a peroxidase-conjugated secondary antibody (Bio-Rad), which were 1:5000 diluted in 5% nonfat dried milk for 1 h. Immunoreactive bands were detected using enhanced chemiluminescence (ECL) reagents or a SuperSignal West Femto Chemiluminescence Substrate (Pierce, Rockford, IL). Membranes were stripped with stripping buffer (50 mM Tris-HCl pH 7.6, 10 mmol/l β-mercaptoethanol and 1% SDS) at 50 °C for 30 min and reprobed with anti-α-Tubulin, anti-SMAD1/5/8, anti-SMAD2, or anti-SMAD3 as a loading control.

siRNA Transfection

To knock down endogenous BMP8A and BMP8B, cells were transfected with 50 nM ON-TARGET*plus*SMART*pool* siRNA targeting BMP8A, BMP8B, or both BMP8A and BMP8B (Dharmacon, Lafayette, CO) using Lipofectamine RNAiMAX (Invitrogen, Life Technologies). The knockdown efficiency of BMP8A and BMP8B was confirmed by RT-qPCR.

Statistical analysis

The results were normalized data from at least three separated experiments. Statistically significant differences between treatments and controls were determined by one-way ANOVA followed by Tukey's test for multiple comparisons of means. Means were considered statistically different if $P< 0.05$ and are indicated by different letters.

A.3 Results

The mRNA levels of BMP2, BMP6, BMP8A and BMP8B, but not BMP5, are detected in SVOG and primary human granulosa cells.

To examine the gene expression of BMP2, BMP5, BMP6, BMP8A and BMP8B in human granulosa cells, the mRNA levels of these BMPs were examined by RT-qPCR in SVOG cells from two continuous generation and primary granulosa cells from two different patients (Figure A-1). The mRNA levels of BMP2, BMP6, BMP8A and BMP8B, but not BMP5, were detected in both SVOG and human granulosa cells. The primary granulosa cells have higher expression levels of BMP6, BMP8A and BMP8B.

The mRNA levels of StAR are not affected by knockdown endogenous BMP8A, but slightly increased by BMP8B knockdown in SVOG cells.

To examine the effects of endogenous BMP8A and BMP8B on StAR expression in human granulosa cells, siRNA (50 nM) were used to knockdown endogenous BMP8A, BMP8B, or both BMP8A and BMP8B for 24 or 48 hours. The knockdown efficiency of BMP8A and BMP8B have been shown in Figure A-2A and A-2B. As shown in Figure A-2C, the mRNA levels of StAR were not affected by BMP8A knockdown while slightly increased by BMP8B knockdown at 48 hours.

The expression of StAR is not changed by treatment with recombinant human BMP8A in SVOG cells

To examine the effects of recombinant human BMP8A on StAR expression, SVOG cells were treated with BMP8A 1, 10, 30 or 100 ng/ml for 24 hours, the mRNA and protein levels of StAR were not affected by BMP8A treatments (Figure A-3).

The expression of ID-1 is not changed by treatment with recombinant human BMP8A in SVOG cells

ID-1 is a well-characterized downstream target of BMPs and BMP canonical SMAD1/5/8 signaling [380]. To examine the effects of recombinant human BMP8A on ID-1 expression, SVOG cells were treated with BMP8A or BMP4 with 30 ng/ml for 3 hours. As shown in Figure A-4, the mRNA levels of ID-1 was increased more than 10-fold by BMP4 treatment, but not affected by BMP8A treatment.

BMP8A has no effect on activation of either canonical SMAD1/5/8 or noncanonical SMAD2/3 signaling in SVOG cells.

To examine whether BMP8A activates canonical SMAD1/5/8 or noncanonical SMAD2/3 signaling in human granulosa cells, SVOG cells were treated with BMP8A 30 or 200 ng/ml, BMP4, TGF-β or Activin A 100 ng/ml for 60 min. As shown in Figure A-5, treatment with BMP8A had no effect on either SMAD1/5/8 or SMAD2/3 activation.

A.4 Figures

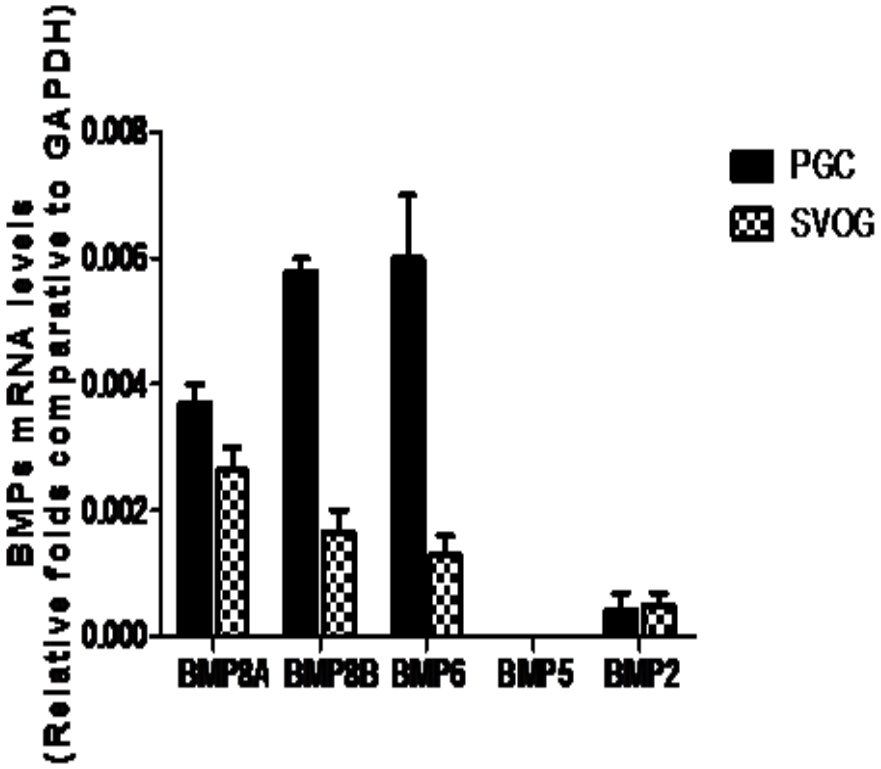

Figure A- 1 The mRNA levels of BMP2, BMP6, BMP8A and BMP8B, but not BMP5, are detected in SVOG and primary human granulosa cells.

The mRNA levels of these BMPs were examined by RT-qPCR in SVOG cells from two continuous generation and primary granulosa cells from two different IVF patients. Relative fold is comparative to GAPDH.

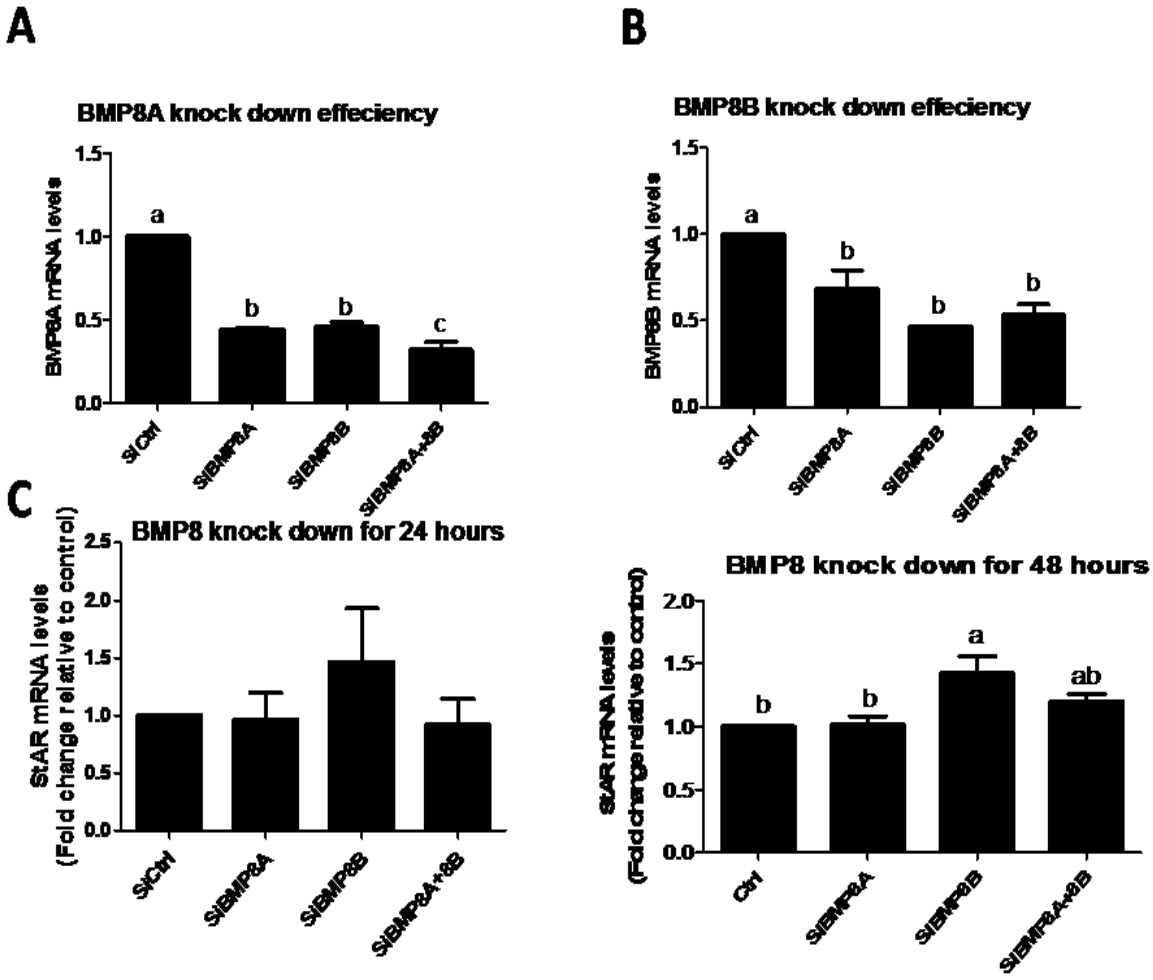

Figure A- 2 The mRNA levels of StAR are not affected by knockdown endogenous BMP8A, while slightly increased by BMP8B knockdown.

SVOG cells were transfected with 50 nM control siRNA (siCtrl), BMP8A siRNA (siBMP8A), BMP8B siRNA (siBMP8B), or both BMP8A and BMP8B siRNA (siBMP8A+8B) for 24 or 48 hours. The mRNA levels of BMP8A (A), BMP8B (B) and StAR (C) were examined by RT-qPCR. Values marked by different letters are significantly different ($p<0.05$).

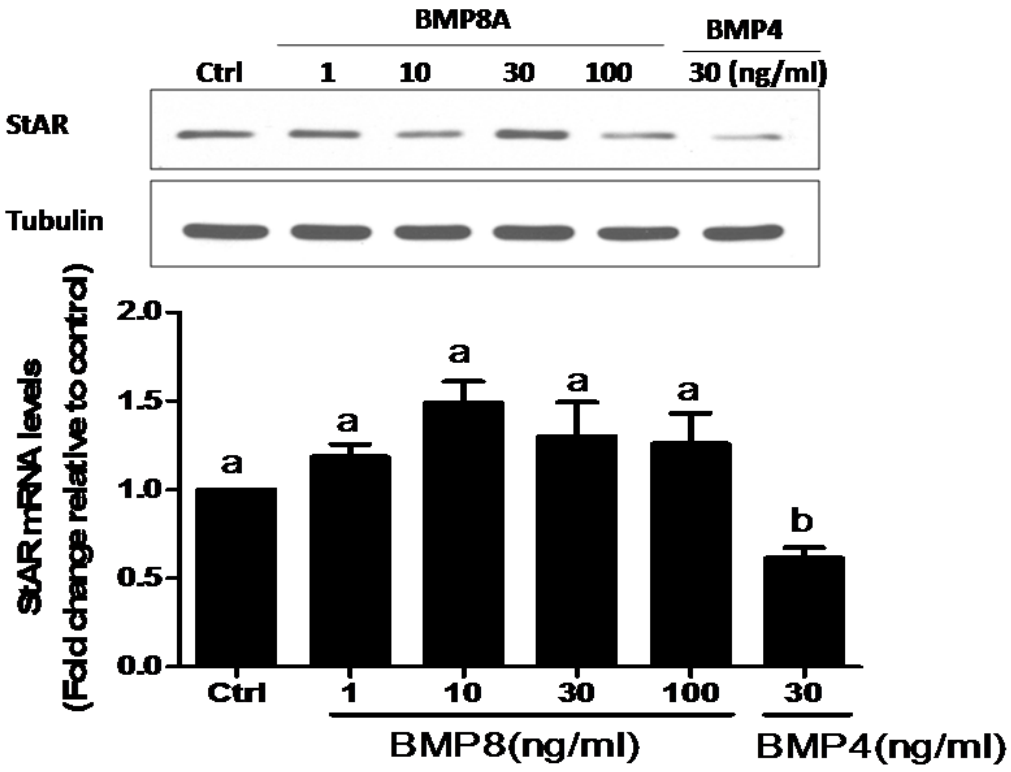

Figure A- 3 The expression of StAR is not changed by treatment with recombinant human BMP8A in SVOG cells

SVOG cells were treated with BMP8A with 1, 10, 30 or 100 ng/ml, or BMP4 30 ng/ml for 24 hours, the mRNA and protein levels of StAR were examined by RT-qPCR and Western blot, respectively. Values marked by different letters are significantly different ($p<0.05$).

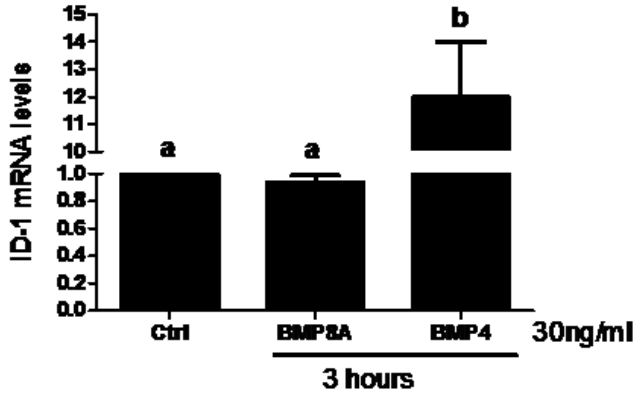

Figure A- 4 The expression of ID-1 is not changed by treatment with recombinant human BMP8A in SVOG cells

SVOG cells were treated with recombinant human BMP8A or BMP4 with 30 ng/ml for 3 hours, the mRNA levels of ID-1 were examined by RT-qPCR. Values marked by different letters are significantly different ($p<0.05$).

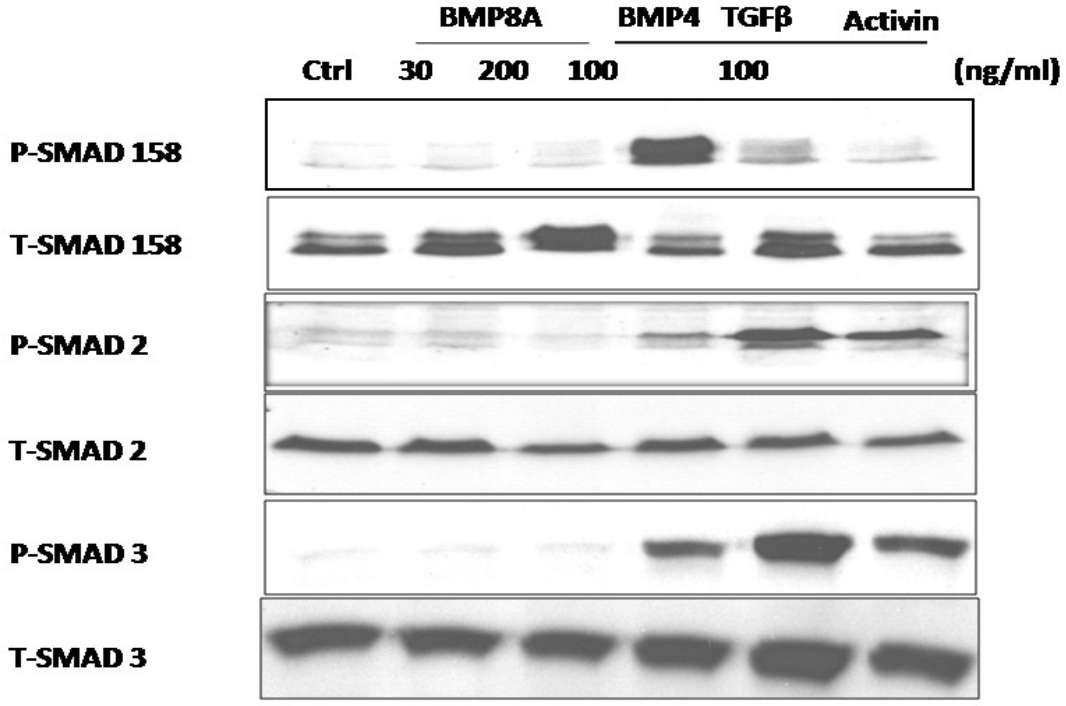

Figure A- 5 Recombinant human BMP8A has no effect on the activation of either canonical SMAD1/5/8 or noncanonical SMAD2/3 signaling in SVOG cells.

SVOG cells were treated with BMP8A 30 or 200 ng/ml, BMP4, TGF-β or Activin A 100 ng/ml for 60 minutes, and the phosphorylated and the basal levels of SMAD1/5/8, SMAD2 and SMAD3 were determined by Western blot.

Appendix B BMP4, BMP6, BMP7, and BMP15 all stimulate COX2 expression in human granulosa cells.

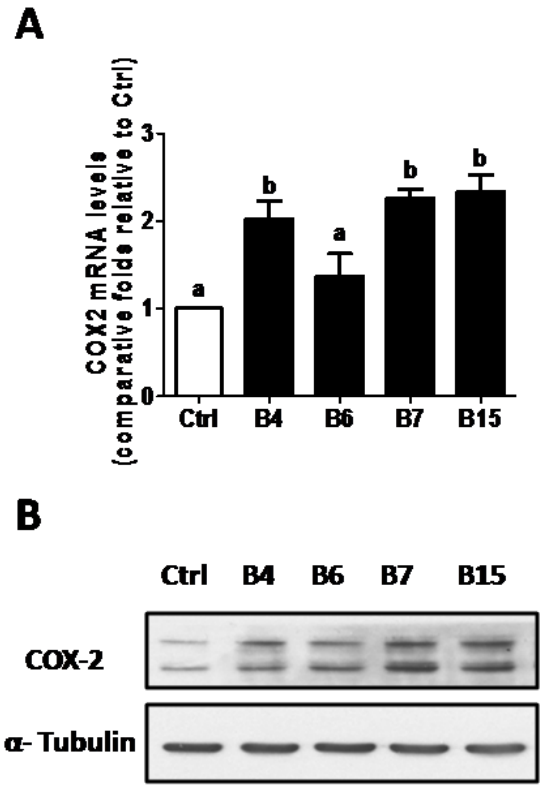

Figure B- 1 BMP4, BMP6, BMP7, and BMP15 all stimulate COX2 expression in human granulosa cells.

SVOG cells were treated with 50 ng/ml of BMP4, BMP6, BMP7 or BMP15 for 3 hours, and the mRNA (A) and protein (B) levels of COX2 were examined by RT-qPCR and Western blot, respectively. Results are expressed as the mean ± SEM of at least three independent experiments.